Univerzita Karlova
Přírodovědecká fakulta

Studijní program: Zoologie
Studijní obor: Zoologie

POHLAVNÍ DIMORFISMUS V OLFAKTORICKÉM SYSTÉMU U MYŠI

Sexual dimorphism in the mouse olfactory system

Disertační práce

Barbora Kuntová

školitel: doc. Mgr. Pavel Stopka, PhD.
konzultant: Mgr. Romana Stopková, PhD.

Praha 2017

Prohlášení

Prohlašuji, že jsem disertační práci vypracovala samostatně, a to s použitím výsledků, které jsem získala společně s řadou spoluautorů, a kteří souhlasí s jejich prezentací formou této práce. Zároveň prohlašuji, že tato práce, ani její část, nebyla použita k získání jiného akademického titulu.

V Praze …… ……..…….

Poděkování

Ráda bych poděkovala svému školiteli Pavlu Stopkovi a jeho ženě Romaně, mé konzultantce, za vedení v průběhu celého doktorského studia. Jejich trpělivý přístup, cenné rady, čas, který mi věnovali, inspirující nápady a bezbřehé znalosti, mě dokázaly celé čtyři roky motivovat v dalším objevování záhadného světa chemické komunikace. Bez nich by tato práce nejspíš nikdy nevznikla.

Dále děkuji svým kolegům a vlastně celému týmu naší laboratoře, jmenovitě pak Martině Černé a Leoně Havrdové za přátelství, vždy veselou náladu, milou společnost a řadu dovedností, které mě naučily. A nakonec velké dík patří naší laborantce, Helence Uhlířové, která mě vždy vyslechla, politovala a poskytla slova útěchy a povzbuzení pro další dny ať už v laboratoři, myšárně či u počítače.

Největší dík však patří mým rodičům, manželovi a dcerce Nelince. Bez podpory rodičů, ať už finanční či morální, bych nikdy nemohla studium na fakultě ani zahájit. Bez podpory manžela bych nedokázala ve studiu pokračovat a současně vychovávat naši holčičku. I jí, ač je jí teprve rok, patří velký dík. Ona je pro mě velkou motivací ženoucí mě vpřed ve snaze být nejen lepší matkou, ale i trpělivým cílevědomým člověkem, který umí svůj čas a energii vložit do správných hodnot.

ABSTRAKT

Pohlavně dimorfní chování myši domácí (*Mus musculus musculus*) je ovlivňováno různými fyzikálními a chemickými podněty. Chemické signály jsou však nejpodstatnější pro individuální rozpoznávání a vyvolávají různé efekty v reprodukčním chování příjemce. Myši patří mezi makrosmatické savce, tudíž je jejich čich velmi dobře vyvinut a jsou schopné rozpoznávat široké spektrum ligandů od ostatních jedinců, ale také z okolního prostředí. Těkavé signály patří mezi organické látky produkované většinou tkání. Mohou mít škodlivý vliv na buňky a proto jsou transportovány ven z těla lipokalinovými transportéry, přičemž některé mohou fungovat jako signály. Tyto těkavé signály jsou schopné stimulovat chemosensorické neurální receptory a tak vyvolávat odpověď v neurálních drahách. Vnímání ligandů má odlišný efekt na samce a samice, ačkoliv dosud nebylo u divoce žijící myši prokázáno, zda jsou tyto rozdíly způsobeny variabilitou v receptorech a neurálních procesech či spíše rozdíly v expresi pohlavně specifických signálů.

Cílem této disertační práce bylo provést srovnávací analýzu orofaciálních tkání a určit specifitu v expresi lipokalinů. Poprvé jsme ukázali, že slzy, nosní sekret a sliny obsahují lipokaliny, které byly původně detekovány pouze v moči. Dokázali jsme, že lipokaliny jsou hojně zastoupené v těchto tkáních a mohou tvořit až 40% z celkového množství proteinů (např. v nosní dutině). Hladina pohlavního dimorfismu v celkovém transkriptomu olfaktorického epitelu a vomeronasálního orgánu je ovšem nízká, naopak hladina pohlavního dimorfismu na úrovni proteinové exprese nosní dutiny je vysoká. Sexuálně dimorfní proteiny patří do různých skupin antimikrobiálních proteinů a lipokalinů. Nejvýznamnějším výsledkem je důkaz, že hladina exprese většiny lipokalinů, a současně jejich individuální variabilita, je závislá na variabilitě antimikrobiálních proteinů a tudíž i na mikrobiotě jedince.

ABSTRACT

Sexually dimorphic behaviourof the house mouse (*Mus musculus musculus*) relies on various physical and chemical cues, however, chemical signals are the most essential cues for individual recognition and in causing various priming effects on reproductive behaviour of the receiver. House mice belongto macrosmatic mammals, and thus,their sense of smell is highly developed and is able to recognize a wide spectrum of ligands from other individuals and from their surrounding environment. Volatile signals belong to organic compounds thatare produced bymost tissues, and may have harmfull effects on cells, and thus they are transported out of the body with lipocalin transporters where some of them may function as signals. These volatile signals are able to stimulate chemosensory neuronal receptors, and thus, yield particularresponses in neural circuits. The ligand sensing has a differential effect upon males and females, however, it has not been shown yet in wild mice whether these differencesare also caused by the variation in receptors and neural processing, or rather by differential expression of signals typical for each sex.

The aim of this thesis was to perform comparative analysis of orofacial mucosal tissues to determine the specificity of expression of particular lipocalins. For the first time we have demonstrated that tears, nasal secretions and saliva contain lipocalins that were previously detected only in the urine. We have provided evidence that the abundance of lipocalins is high in these tissues and may account for as much as 40% of all proteins detected (for example in the nasal cavity). Interestingly, the level of sexual dimorphism in the expression of all transcripts in olfactory epithelia and vomeronasal organ is low, whilst the level of dimorphism in protein expression in the nasal cavity is high. However, these sexually dimorphic proteins belong to various families of anti-microbial proteinsand also include lipocalins. The most interesting result of my thesis is evidence that the level of expression of most lipocalins and their individual variation is driven by the variation in antimicrobial proteins and thus presumably depends on particular microbiota of individual mice.

Použité zkratky

zkratka	anglicky	česky
ABPs	androgen binding proteins	androgen-vázající proteiny
AOB	accessory olfactory bulb	přídatný čichový lalok
APOD	apolipoprotein D	apolipoprotein D
BTP	beta trace protein	
DHB	3,4-dehydro-exo-brevicomin	3,4-dehydro-exo-brevikomin
DMP	2,5-dimethylpyrazine	2,5 - dimetylpyrazin
ESPs	exocrine gland-secreted peptides	exokrinní sekreční peptidy
FABPs	fatty acid-binding proteins	mastné kyseliny vázající proteiny
FPR	formyl peptide receptor	-
GC-D	guanylyl cyclase D receptors	-
GPCRs	G-protein coupled receptors	receptory spřažené s G-proteiny
HDL	high density lipoprotein	vysokodenzitní lipoprotein
HMH	6-hydroxy-6-methyl-3-heptanon	6-hydroxy-6-metyl-3-heptanon
LCNs	lipocalins	lipokaliny
LG	lacrimal gland	lakrimální žláza
L-PGDS	lipocalin-type-prostaglandin-D-synthase	
MOB	major olfactory bulb	hlavní čichový lalok
MOE	major oflactory epitel	hlavní čichový epitel
MTMT	(metylthio)metanethiol	(metylthio)metanetiol
MUPs	major urinary proteins	hlavní močové proteiny
NALT	nasal-associated lymphoid tissue	NALT
NGAL	neutrophil gelatinase-associated receptor	
ORs protein	olfactory receptors	olfaktorické receptory
OBPs	odorant binding proteins	odoranty-vázající proteiny
Olfr gen	olfactory receptor	olfaktorický receptor
PBP	pyrazin binding protein	pyrazin-vázající protein
PGRMC1	progesterone receptor membrane component1 protein	
RBP	retinol binding protein	retinol-vázající protein
ROS	reactive oxygen species	reaktivní formy kyslíku
SCGBs	secretoglobins	sekretoglobiny

SBT	2-sec-butyl-dihydrothiazol	2-sec-butyl-dihydrothiazol
SMG	submandibular gland	submandibulární žláza
TRPC2	Transient receptor potential cation channel, subfamily C, member 2	
V1R protein	vomeronasal receptor type 1	V1 receptor
V2R protein	vomeronasal receptor type 2	V2 receptor
Vmnr gen	vomeronasal receptor	vomeronasální receptor
VNO	vomernasal organ	vomeronasální orgán
VSNs	vomeronasal sensory neurons	vomeronasální sensorické neurony

OBSAH

1 ÚVOD

Smyslové vnímání okolního prostředí se liší v závislosti na pohlaví jedince. U člověka je dominantním smyslem zrak, přičemž zde existují rozdíly ve vnímaní barevného spektra mezi jedinci opačného pohlaví. Stejně tak se odlišuje sensitivita vůči chemickým signálům, tedy odorantům, feromonům a chuťovým podnětům, ačkoliv je spektrum olfaktorických signálů, které člověk jakožto mikrosmatický savec rozlišuje, v porovnání s makrosmatickou myší, zanedbatelný.

Evoluční význam schopnosti vnímat chemické látky odráží u obratlovců variabilní škála chemosensorických tkání, neuronů a genů. Savci obecně disponují dvěma olfaktorickými systémy, a to hlavním čichovým epitelem a vomeronasálním orgánem (u lidí chybí, ale nalezen jeden vomeronasální receptor). Hlodavci mají navíc septální orgán Masera a Grünebergův ganglion, čímž ještě vylepšují už tak vysokou senzitivitu čichového aparátu. Tyto orgány exprimují různé receptory, jejichž ligandy jsou těkavé odoranty i látky feromonální povahy (Buck & Axel, 1991; Jia et al., 1996). Po vazbě těchto ligandů na receptor jedinec dostává informaci o dostupné potravě, přítomnosti predátora, přítomnosti partnera k páření či o dominanci samce.

Feromony jsou molekuly různé chemické povahy, které u recipienta vyvolávají odpovídající chování či změnu fyziologického stavu. U myší jsou feromony hojně přítomny v moči, zejména u samců, a protože jsou ve vodním prostředí nestabilní, využívají proteinových transportérů z rodiny lipokalinů, které je ochraňují a zpomalují jejich uvolňování do vnějšího prostředí (Timm et al., 2001; Sharrow et al., 2002). Lipokaliny, které mají ve své terciární struktuře beta barel, představují ideální kandidáty pro vazbu feromonálních ligandů. Z hlediska chemické komunikace jsou nejstudovanějšími lipokaliny tzv. hlavní močové proteiny (angl. major urinary proteins - MUPs), ale patří sem i další jako odorant vázající proteiny (angl. odorant binding proteins - OBPs) a lipokaliny (angl. lipocalins - LCNs). Všechny tyto skupiny lipokalinů jsou exprimovány v různých tkáních a plní variabilní funkce, od transportu signálu, přes imunitní funkce až po detoxifikaci (Pevsner et al., 1990; Pevsner & Snyder 1990; Flower et al., 2000; Flo et al., 2004; Grolli et al., 2006; Kwak et al., 2016; Stopková et al., 2016).

Cílem této práce je charakterizovat pohlavní dimorfismus v expresi lipokalinů (MUPs, OBPs, LCNs) a dalších kandidátních proteinů účastnících se chemické komunikace, a to jak na úrovni transkriptomu, tak i proteomu, ve tkáních a sekretech

orofaciální oblasti. Rozdíly v expresi transportérů feromonů mezi samci a samicemi by mohly vysvětlit podstatu pohlavně specifických projevů v chování, které jsou vyvolány olfaktorickými signály, a podílet se tak na sexuální signalizaci.

2 OLFAKCE A CHEMICKÁ KOMUNIKACE

Chemická komunikace,zprostředkovaná chemosignály z okolního prostředí, se vyskytuje u všech organismů, již na úrovni jednobuněčných, nezávisle na prostředí. S postupující evolucí se mechanismy uvolnění, příjmu, zpracování a vyhodnocování signálů stávaly složitějšími a důmyslnějšími, diversifikovaly se tkáně a orgány, organizovala mozková centra. Olfakce neboli čich hraje u mnoha druhů živočichů nezastupitelnou, životně důležitou roli a je u nich tudíž velmi dobře vyvinut (makrosmatické druhy). U jiných druhů naopak zastává pouze roli minoritní (mikrosmatické druhy). Myši (rod *Mus*) jsou makrosmatičtí savci, čich je u nich vyvinut k dokonalosti, a jsou schopné detekovat obrovské množství variabilních chemických signálů. Do percepce pachových molekul (feromon / odorant) informujících o okamžitém stavu okolního prostředí se zapojuje kaskáda proteinových transportérů (např. hlavní močové proteiny - angl. major urinary proteins - MUPs), specifických receptorů (vomeronasální / čichový receptor) a signálních drah několika percepčních orgánů (např. vomeronasální orgán). Zpracování vjemů pak probíhá dle typu signálu v různých částech mozku (např. hlavní a přídatný čichový lalok) a následné reakce formou specifického chování či změny fyziologického stavu jsou též evokovány rozdílně. Právě vnitrodruhová pachová komunikace a sensitivita vůči chemosignálům z okolního prostředí moduluje sociální interakce myší, určuje úspěšnost nalezení potravy či sexuálního partnera, zajišťuje rozpoznávání příbuzných (kin recognition), navigaci, rodičovské a agresivní chování, ale také včasné varování před blízkostí predátora (Novotný et al., 1985; Hurst et al., 1993; Chamero et al., 2007, Roberts et al., 2010).

2.1 Chemický signál

Chemické signály jakožto prvotní podněty čichového ústrojí nelze jednoznačně charakterizovat. Může se jednat např. o jednoduché aminy, aromatické sloučeniny, terpeny či dokonce netěkavé peptidy. Obecně se rozdělují na odoranty a feromony, ale vymezení se překrývá. Původně pojem "feromon" označuje chemickou látku produkovanou jedincem jednoho druhu, která svým působením vyvolá specifické chování či fyziologickou odpověď pouze u příjemce stejného druhu (Karlson & Luscher, 1959). Vzhledem k účinku se pak feromony dělí na "releaser" feromony s okamžitým efektem na chování (př. agrese, páření, značkování teritoria) a "primer" feromony ovlivňující reprodukci z dlouhodobějšího hlediska. Příkladem může být tzv. Whitten efekt, kdy přítomnost samčího pachu indukuje u samic estrus (Whitten et al., 1968). Později se však ukazuje, že jednotlivé feromony samy o sobě nemají takový účinek jako celková skladba feromonů (angl. pheromonal blend) v pachu. Každý z nich může mít různé účinky na reprodukci a chování, zároveň dva různé mohou vyvolat stejný efekt, a dokonce ani nemusí být druhově specifické (Jemiolo et al., 1986; Rasmussen et al., 1996). Feromony mohou být volatilní (těkavé) či netěkavé látky.

Na druhou stranu odoranty zahrnují látky pocházející z okolního prostředí, těkavé, které informují jedince o blízkosti potravy či nebezpečí. Chemické signály sloužící k interspecifické komunikaci lze rozdělit na alomony a kairomony. Alomony jsou takové signály, které poskytují výhodu svému producentu, např. rostliny ochraňují před parazitickým hmyzem. Kairomony naopak přinášejí benefit příjemci, např. pach predátora varuje případnou kořist. Reakce na tyto chemosignály jsou okamžité, kdy se zvíře rozhoduje např. pro útěk či pro pokračování v hledání zdroje odorantu.

2.2 Chemosensorické orgány a receptory

Chemické signály, se kterými se organismus ve svém okolí setká, ať už odoranty či feromony, se skrze nosní dutinu dostávají k receptorům, jejichž neurony vedou informace dál do specifických částí mozku. Na percepci chemických signálů se u většiny obratlovců podílejí dva separované orgány, hlavní čichový epitel (angl. major olfactory epithelium - MOE) a vomeronasální orgán (angl. vomeronasal organ -

VNO). Hlodavci mají navíc dvě prostorově oddělená seskupení chemosensorických neuronů označovaných jako Grünebergův ganglion a septální orgán Masera.

Všechny zmíněné orgány k percepci signálů využívají některé z několika typů chemo-sensorických receptorů: olfaktorické receptory (angl. olfactory receptors - ORs), vomeronasální receptory (angl. vomeronasal receptors - VRs), TAAR receptory (angl. trace amine-associated receptors) a FPR receptory (angl. formyl peptide receptors) (review Hayden & Teeling, 2014). Všechny typy těchto receptorů patří mezi transmembránové proteiny se sedmi doménami a jsou spřažené s G-proteiny (angl. G protein-coupled receptors - GPCRs).

Původní hypotéza, že olfaktorické receptory hlavního čichového epitelu jsou výlučnými recipienty odorantů, kdežto vomeronasální receptory feromonů, je již překonána. V současnosti je již známo, že některé látky s feromonálními účinky jsou detekovány v MOE a naopak, že odoranty mají své receptory ve VNO. Percepce variabilních chemosignálů se tedy v MOE a VNO překrývá a kooperace obou systémů funguje na různých úrovních (Restrepo et al., 2004; Levai et al., 2006; Xu et al., 2005).

2.2.1 Hlavní čichový epitel (MOE)

MOE je lokalizován v zadní části nosní dutiny, kde pokrývá chrupavku. Receptory na sensorických neuronech se do kontaktu s ligandy, tedy těkavými odoranty (resp. feromony), dostávají pasivně s vdechovaným vzduchem. Mezi ORs patří obsáhlé množství proteinů, tvořící vůbec nejpočetnější genovou skupinu u obratlovců. Repertoár ORs u myši zahrnuje okolo 1300 genů, z čehož 20% tvoří pseudogeny, rozdělených do 228 proteinových rodin (Zhang & Firestein, 2002). ORs jsou receptory spřažené s G-proteiny, konkrétně s $G\alpha_{olf}$ podjednotkou. Axony sensorických neuronů směřují do hlavního čichového laloku (angl. major olfactory bulb - MOB) a odtud jsou informace směřovány do vyšších mozkových center.

Každý olfaktorický neuron nese jeden typ OR, přičemž každý odorant je rozeznáván několika ORs a zároveň jeden OR rozeznává několik odorantů. Princip detekce chemických signálů v MOE tak spočívá na kombinování receptorů tvořících určitý kód. Různé odoranty mají různé kombinace ORs (Buck & Axel, 1991). Kromě klasických odorantů ORs pravděpodobně detekují i některé MHC peptidy (Spehr et al., 2006).

Zajímavé je, že některé ORs jsou exprimovány ve tkáních, které nejsou chemosensorické. Prvním místem ektopické detekce ORs byla varlata a maturované spermie. Spermie v *in vitro* podmínkách reagují chemotakticky na testované syntetické chemosignály a předpokládá se, že skrze ORs detekují molekuly, které vedou ke změně koncentrace intracelulárního Ca^{2+} (Parmentier et al., 1992, Spehr et al., 2003). Dalšími místy exprese ORs jsou ledviny, srdce, mozek, placenta, primordiální zárodečné buňky, buňky červené krevní řady, atd. Funkce ORs v těchto tkáních je ovšem zatím stále nejasná, uvažuje se o účasti v morfogenetických procesech, rozpoznávání buněk apod. (review Kang & Koo, 2012).

MOE navíc obsahuje minimálně další tři typy receptorů detekujících různé substance. TAARs byly v roce 2006 objeveny jako skupina receptorů specifická pro MOE (Liberles & Buck, 2006). Ačkoliv mají blíže k receptorům pro biogenní aminy (např. serotoninový receptor), patří stejně jako ORs mezi receptory spřažené s G-proteiny. U myši je popsáno 15 členů exprimovaných na minoritní populaci neuronů MOE. Každý z nich váže specifický set malých aminů přítomných třeba v myší moči, např. isoamylamine, trimethylamine and *β*-phenylethylamine (Zucchi et al., 2006). Předpokládá se, že tyto molekuly mají vliv na sociální status, např. při urychlení nástupu puberty myších samic (Nishimura et al., 1989). Další specifitou MOE jsou GC-D receptory (angl. guanylyl cyclase D receptors) exprimované na neuronech lokalizovaných v dorsální části MOE a aktivující speciální glomeruly v hlavním čichovém laloku, tzv. "necklase glomeruli". Jejich ligandy a funkce jsou zatím nejasné (Fulle et al., 1995). V MOE se vyskytuje také jeden typ vomeronasálních receptorů, konkrétně V1R (angl. vomeronasal receptor type 1), uplatňující se v detekci těkavých feromonů (Karunadasa et al., 2006).

2.2.2 Vomeronasální orgán (přídatný čichový orgán, Jacobsonův orgán; VNO)

Poprvé byl vomeronasální orgán popsán v roce 1813 anatomem L. Jacobsonem jako sekreční orgán v nose savců (chybí u starosvětských opic, mořských savců, lidoopů a lidí). VNO je bilaterálně symetrický, cylindrický orgán uzavřený v kostěné kapse v přední části nosního septa. Na rozdíl od MOE, kam jsou odoranty přinášeny pasivně společně s vdechovaným vzduchem, feromony do VNO musí být dopravovány aktivně. To vyžaduje přímý kontakt se zdrojem a tzv. pumpováním

(dáno autonomně řízenou vazokonstrikcí a vazodilatací přiléhajících cév) se feromony dostávají do lumen VNO k sensorickým neuronům (Meredith &O´Connell, 1979). Detekce feromonů je ve VNO rozdělena do dvou anatomicky oddělených částí, apikální a bazální. Ty se liší expresí receptorů a asociovaných G-proteinů na svých sensorických neuronech. Rozdělený je i přídatný čichový lalok (ang. accessory olfactory bulb - AOB), kam vomeronasální sensorické neurony (angl. vomeronasal sensory neurons - VSNs) vysílají své axony (Jia et al., 1996, 1997).

Prvními objevenými VNO receptory byly V1R receptory (angl. vomeronasal receptor type 1 - V1Rs) (Dulac & Axel, 1995) asociované s $G\alpha_{i2}$. Jsou exprimovány na apikální části VNO a své axony směřují k anteriorní oblasti AOB (Jia et al., 1996, 1997). Myši mají téměř 200 typů V1Rs a ty se rozdělují na 12 proteinových rodin (Rodriguez et al., 2002). Exprese V1Rs receptorů je monogenní a monoalelická, tudíž na jedné sensorické buňce je vždy pouze jeden typ receptoru. V1Rs tak velmi specificky váží malé organické molekuly, volatilní feromony a odoranty. V2R receptory (angl. vomeronasal receptor type 2 - V2Rs) byly objeveny až v roce 1997 (Matsunami & Buck, 1997) a u myší čítají přibližně 120 funkčních typů rozdělených do 4 čeledí označovaných A-D. Asociovány jsou s jiným typem G-proteinu, a to s $G\alpha_o$ v bazální části VNO. Axony těchto sensorických neuronů pak směřují k posteriorní oblasti AOB (Jia et al., 1996, 1997). Na rozdíl od apikálních neuronů, na jednom bazálním sensorickém neuronu jsou exprimovány dva typy V2Rs receptorů, přičemž jeden z nich vždy patří do C čeledi (Martini et al., 2001). Uvádí se, že díky velké extracelulární podjednotce mohou tyto receptory vázat i větší peptidy, např. MHC peptidy 1. třídy (Leinders-Zufall et al., 2004) a ESP1 (Exocrine gland secreted peptide 1) (Kimoto et al., 2005).

Princip rozpoznání konkrétního feromonu není pravděpodobně univerzální jako je tomu u ORs. Původní výzkum ukazuje na velkou senzitivitu a selektivitu V1Rs i V2Rs v rozeznávání feromonů. Tvrdí, že každá látka aktivuje unikátní set sensorických neuronů a vytváří tak určitý "vzorec", který je nezávislý na koncentraci ligandu (Leinders-Zufall et al., 2000).

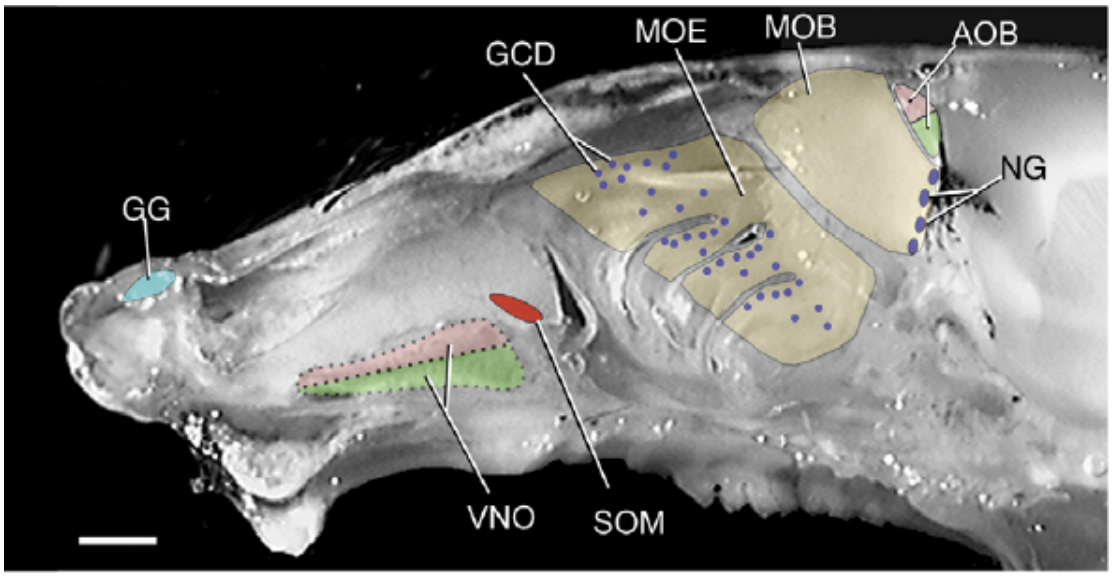
Malá podskupina neuronů v apikální části VNO exprimuje navíc pro VNO unikátní FPR receptory (formyl peptide receptors). Jsou to s receptory spřažené s G-proteiny a v myším VNO je pět typů FPR. Ligandy těchto receptorů jsou různé molekuly vzniklé degradací bakterií. Nepřímo tedy mohou mít funkci ochranné antimikrobiální bariéry (Rivière et al., 2009).

Ve VNO jsou exprimovány i ORs, jejichž axony směřují stejně jako axony VSNs do AOB. Ty odpovídají za schopnost VNO detekovat i běžné odoranty, nejen feromony (Levai et al., 2006).

Jak již bylo zmíněno, chemická komunikace řídí řadu sociálních interakcí. Substance, které se účastní těchto interakcí, nejsou vždy známy a stejně tak ani jejich receptory. Pro získání celkového přehledu o okolí jakožto směsi pachů, existuje v percepci chemosignálů MOE a VNO překryv: feromony (těkavé) i odoranty jsou detekovatelné oběma olfaktorickými systémy (Xu et al., 2005; Restrepo et al., 2004; Levai et al., 2006). Tato vzájemná spolupráce a komplementarita vedla k hypotéze, že prvotní vjemy získané s proudem vzduchu a vyhodnocené recipientem v MOE jako zajímavé, podpoří aktivní explorativní chování myši a následnou aktivaci VNO pumpy (Keverne et al., 2004).

Komplementarita mezi olfaktorickými tkáněmi existuje na úrovni transdukce signálů do mozku. Například neurony z anteriorní oblasti hypotalamu, které řídí pohlavní chování a kopulaci, přijímají signály jak z OSNs, tak VSNs (Boehm et al., 2005). Utváří se tak komplexní informace o pachu v okolí jedince.

Obr.1. Olfaktorický systém myši (převzato z Zufall & Leinders-Zufall, 2007)



AOB - vedlejší čichový lalok; GCD - guanylyl cykláza D receptory; GG - Grünebergův ganglion; MOB - hlavní čichový lalok; MOE - hlavní čichový epitel; NG -necklase glomeruli; SOM - septální orgán Masera; VNO- vomeronasální orgán

Tab. 1. Přehled receptorů a ligandů v olfaktorických tkáních

Receptor	Zkratka/Typ	Ligand	Orgán
Olfaktorický	OR	odoranty a feromony	hlavní čichový epitel vomeronasální orgán Septální orgán Masera
Trace amine-associated	TAAR	volatilní aminy	hlavní čichový epitel Grünebergův ganglion
Vomeronasální	V1R	odoranty a feromony	vomeronasální orgán hlavní čichový epitel
	V2R	nevolatilní feromony	vomeronasální orgán Grünebergův ganglion
	FRP	bakterie,proteiny mitochondrií a zánětu	vomeronasální orgán

2.2.3 Další olfaktorické subsystémy

V oblasti nosní dutiny hlodavců jsou další minoritní tkáně obsahující receptory pro detekci chemosignálů. Grünebergův ganglion (GG) je lokalizován v přední části. Neurony tohoto chemosensorického orgánu se spojují a každý svazek směřuje do jednoho z přibližně 10 glomerulů v MOB, vytváří zde tzv. necklace glomeruli (NG). GG exprimuje 2 typy receptorů, TAARs a V2R (Fleischer et al., 2006; 2007), které pravděpodobně detekují intraspecifické alarm feromony či kairomony (Brechbühl et al., 2008).

Septální orgán Masera je lokalizován na bázi nosního septa jako samostatný ostrůvek olfaktorického epitelu. Neurony exprimují ORs a směřují do glomerulů v zadní části MOB (Ma et al., 2003). Vzhledem k umístění může orgán fungovat jako primární detektor biologicky relevantních molekul uvolňovaných např. při olizování (Tirindelli et al., 2009).

2.3 Signální transdukce a mozková centra asociovaná s olfakcí

S existencí různých olfaktorických sensorických neuronů, a tedy i receptorů, existuje variabilita i v systému druhých poslů a iontových kanálů, které transformují vazbu ligandu na receptor v elektrický signál. V případě ORs, $G\alpha_{olf}$ podjednotka aktivovaná po vazbě odorantu spustí syntézu cyklického AMP (cAMP)adenylyl cyklásou 3. Zvýšená koncentrace cAMP pak způsobí otevření CNG kanálu (cyclic nucleotide-gated channel), následuje influx Na^+ a Ca^{2+} iontů, otevření chloridových kanálů a eflux Cl^- depolarizuje membránu neuronu (Kurahashi & Menini, 1997).

U vomeronasálních receptorů se po vazbě ligandu aktivuje βγ podjednotka G-proteinu a ta stimuluje fosfolipázu C k produkci lipidického diacylglycerolu (DAG) a inositoltrisfosfátu (IP3). Otevření TRPC2 kanálu (transient receptor potential cation channel 2) a influx Na^+ a Ca^{2+} vede k depolarizaci membrány. TRPC2 je specifický pro vomeronasální receptor (Liman et al., 1999) a esenciální pro sexuální a sociální chování myší (Leypold et al., 2002; Stowers et al., 2002). Samci deficientní v Trpc2 nejsou schopni rozlišovat pohlaví a je u nich redukováno vzájemné agresivní chování, u samic je potlačeno chování v průběhu laktace a mateřství. Překvapivě však navíc mutantní samice vykazují znaky typické pro samčí chování, jako je vokalizace, páření, nadbíhání samicím. To je dáno tím, že neurální dráhy, které řídí chování u samců jsou zachovány i u samic. Výsledné chování je však závislé na pohlavně specifických senzorických podnětech VNO, které u samic potlačují samčí a naopak udržují samičí a mateřské chování (Kimchi et al., 2007).

Po depolarizaci membrány signál z receptoru putuje po axonu dál do glomerulů v čichových lalocích. Senzorické neurony MOE exprimující jeden stejný receptor se sbíhají do stejného glomerulu v MOB (Mombaerts et al., 1996). Z glomerulů vedou signál mitrální buňky do primárního olfaktorického kortexu složeného z několika odlišných částí (např. kortikální amygdala, piriform kortex). Dále je informace šířena do hypotalamu, thalamu a dalších částí (rev. Shipley & Ennis, 1996).

U VRs je situace odlišná, do jednoho glomerulu se sbíhají axony VSNs exprimujících různé receptory a jeden VR směřuje dendrity do většího množství malých glomerulů v AOB (Rodriguez et al., 1999). Mitrální buňky pak směřují axony do oblastí limbického systému, konkrétně do mediální a kortikální amygdaly ("vomeronasální amygdala"), přídatného olfaktorického traktu a *stria terminalis*. Hypotalamus integruje informace z limbického systému v oblasti ventromediální

a mediální preoptické oblasti, které se účastní reprodukčního a sociálního chování (rev. Shipley & Ennis, 1996).

Pro shrnutí, mediální amygdala přijímá signály z MOB i AOB, ale k integraci dochází v basální oblasti telencefalonu.

3 LIPOKALINY

Aby se feromon či odorant dostal ke svému receptoru v olfaktorické tkáni a nepodlehl degradaci nedetekován, využije transportního proteinu. Lipokaliny se svou terciární strukturou tvořící barel, jsou pro vazbu takovýchto ligandů jedineční kandidáti.

3.1 Obecná charakteristika lipokalinů

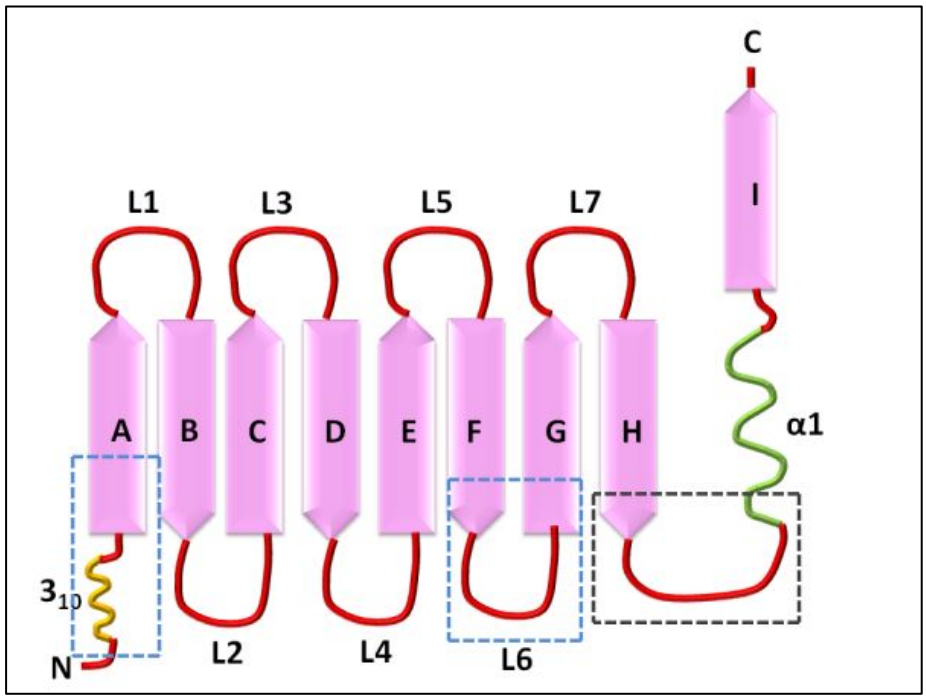
Pojem "lipokaliny" zahrnuje rozsáhlou rodinu proteinů exprimovaných u různých druhů organismů, včetně bakterií (Bishop et al., 1995), rostlin (Charon et al., 2005), hmyzu a obratlovců. Jsou to malé sekreční proteiny schopné vázat hydrofobní molekuly a fungovat tak jako transportéry. Široká škála ligandů, např. feromony a odoranty, retinol, mastné kyseliny, lipidy, biliny a steroidy umožňují uplatnění lipokalinů v různých biologických procesech. U myši lipokaliny čítají 55 kódujících genů.

Lipokaliny patří spolu s avidiny a mastné kyseliny-vázajícími proteiny (angl. fatty acid-binding proteins - FABPs) do nadrodiny tzv. calycinů. Spojovacím znakem této skupiny je terciární struktura utvářející β-barel, v jehož vnitřku jsou vázány ligandy. U lipokalinů tvoří barel osm antiparalelně poskládaných β-listů vymezený N-terminalní 3_{10} α-helixem a C-terminálním α-helixem následovaným jedním β-listem. Konzervativních je i několik dalších strukturních motivů, které vyplívají z amino-kyselinové sekvence. Podle počtu sdílených se dělí na "kernel" lipokaliny (3 motivy) a "outlier" lipokaliny (2 motivy), přičemž GxW motiv je jediný společný pro všechny. Celkově je ovšem sekvenční identita velmi nízká, často méně než 20% (Flower et al., 1993; 1996), přičemž jsou to poměrně malé proteiny čítající průměrně 200

aminokyselin a 20kD. Některé z nich ovšem vytváří oligomery, např. dimer kravského OBP (Tegoni et al., 1996) či oktamery krustakyaninu korýšů (Keen et al., 1991).

U myši je většina lipokalinů lokalizována na chromozomech 2 (př. lipokaliny - *Lcn*s), 4 (*Mup*s) a X (Odorant-vázající proteiny - *Obp*s) (Salier, 2000).

Obr.2. Schéma terciární struktury lipokalinů (převzato z Chakraborty et al., 2011)



Typická struktura lipokalinů tvořená 8 antiparalelními β-listy spojenými smyčkami, N-terminálním 3_{10} α-helixem a C-terminalním α-helixem následovaným jedním β-listem. Oblasti ohraničené v modrých boxech jsou strukturálně konzervované, v černém boxu je oblast s konzervativní aminokyselinovou sekvencí.

3.2 Funkční variabilita savčích lipokalinů

Ačkoliv lipokaliny především transportují své ligandy na místo určení, mohou mít i další funkce, ve kterých jsou nezastupitelní a pro organismus esenciální. Klasický transportér je např. retinol-vázající protein (retinol-binding protein - RBP) chránící retinol před oxidací a usnadňující jeho transport z jater do cílových buněk v periférních tkáních (Blomhoff et al., 1990). Stejně tak apolipoprotein D (APOD), který váže nejčastěji vysokodenzitní lipoprotein (angl. high density lipoprotein - HDL), ovšem škála možných ligandů je veliká v závislosti na tkáni, ve které je

exprimován. Sekundární funkce APOD tak zahrnuje řadu procesů od regenerativních v centrální nervové soustavě (Rassart et al., 2000) přes ochranu před oxidativním stresem v mozku (Ganfornina et al., 2008) až po buněčné dělení (Provost et al., 1991). APOD je také dle fylogenetické analýzy považován za předchůdce všech obratlovčích i hmyzích lipokalinů (Bishop et al., 1995).

Duální funkci má prostaglandin D-syntáza (angl. lipocalin-type-prostaglandin-D-synthase - L-PGDS; beta trace protein - BTP). Jako enzym katalyzuje konverzi prostaglandinu H2 v prostaglandin D2. Jako transportér poskytuje ochranu před buněčným oxidativním stresem tím, že vychytává reaktivní formy kyslíku (angl. reactive oxygen species-ROS) a vynáší je do extracelulárního prostředí (Fukuhara et al, 2012). V humánní medicíně pak funguje jako kardiovaskulární a nefrologický marker (White et al., 2015).

Řada dalších lipokalinů má důležité postavení v imunitním systému. Zásadní roli v nespecifické imunitní odpovědi na infekci má Lipocalin 2 (LCN2; Siderocalin; neutrophil gelatinase-associated receptor - NGAL), a to díky schopnosti vázat bakteriální siderofory (katecholátní typ), které tvoří zásobu železa pro růst Gram-negativních bakterií (Flo et al., 2004). Podobné schopnosti má i lidský a potkaní Lipocalin 1 (LCN1, tear lipocalin), ovšem váže odlišný typ sideroforu (hydroxymátní typ) a navíc je účinný v antioxidativní ochraně vychytáváním produktů peroxidace lipidů (Fluckinger et al., 2004). Funkce ROS detoxifikace byla popsána i u OBP, konkrétně u kravského a prasečího OBP (Grolli et al., 2006). Orosomucoid, protein akutní fáze inflamace, funguje jako inhibiční imunomodulátor řady dějů zahrnutých v procesu hojení zranění - agregace destiček, chemotaxe neutrofilů, proliferace lymfocytů apod. (review Ligresti et al., 2012).

Lipokaliny plní řadu funkcí i v reprodukčním systému savců. U koní, konkrétně kobyl, je specifický uterokalin, protein exprimovaný děložní sliznicí s předpokládanou funkcí v udržování gravidity (Crossett et al., 1996). Zajímavým lipokalinem u lidí a opic je glykodelin (pregnancy-associated endometrial alpha-2 globulin; PAEG; PP14), vyznačující se variabilitou v posttranslační glykosylaci v závislosti na pohlaví. Vznikají tak dvě funkčně odlišné formy glykodelinu. Samičí glykodelin-A je exprimován endometriem, vaječníky a vejcovody. Ve vysoké koncentraci je přítomen v amniotické tekutině v prvním trimestru těhotenství a pravděpodobně se podílí na potlačení aktivity matčina imunitního systému vůči plodu. Glykodelin-S je obsažen v seminální plasmě samců. Má schopnost přímé interakce se spermiemi. Navázáním

na hlavičku spermie inhibuje vazbu na *zona pellucida* vajíčka (review Seppälä et al., 2002).

Myší LCN3 (Vomeronasal secretory protein 1 - VNSP1) a LCN4 (Vomeronasal secretory protein 2 - VNSP2) jsou lipokaliny exprimované výhradně VNO a posteriorními žlázami nasálního septa. Předpokládáná funkce je transport signálních molekul k VNO sensorickým neuronům (Miyawaki et al., 1994). V myším nadvarleti je exprimován LCN5 (mouse epididymal retinoic acid binding protein - mERABP), který váže retinoovou kyselinu a svou asociací se spermiemi se tak nepřímo podílí na maturaci (Lareyre et al., 1998). Gen pro *Lcn5* byl spolu s dalšími lipokaliny, konkrétně *Lcn 8, Lcn 9, Lcn 10, Lcn 12* a *Lcn13*, popsán jako gen specificky exprimovaný v nadvarlatech (Suzuki et al., 2004). Ovšem později byl např. LCN13 detekován v řadě jiných tkání jako je slinivka, kosterní svaly a v játrech, a předpokládá se, že má roli v metabolismu glukózy (Cho et al., 2011). V našich výzkumech jsme LCN13 detekovali v proteomu slin, nosu a slz (Stopka et al., 2016; Kuntová et al., 2017; Stopková et al., 2017).

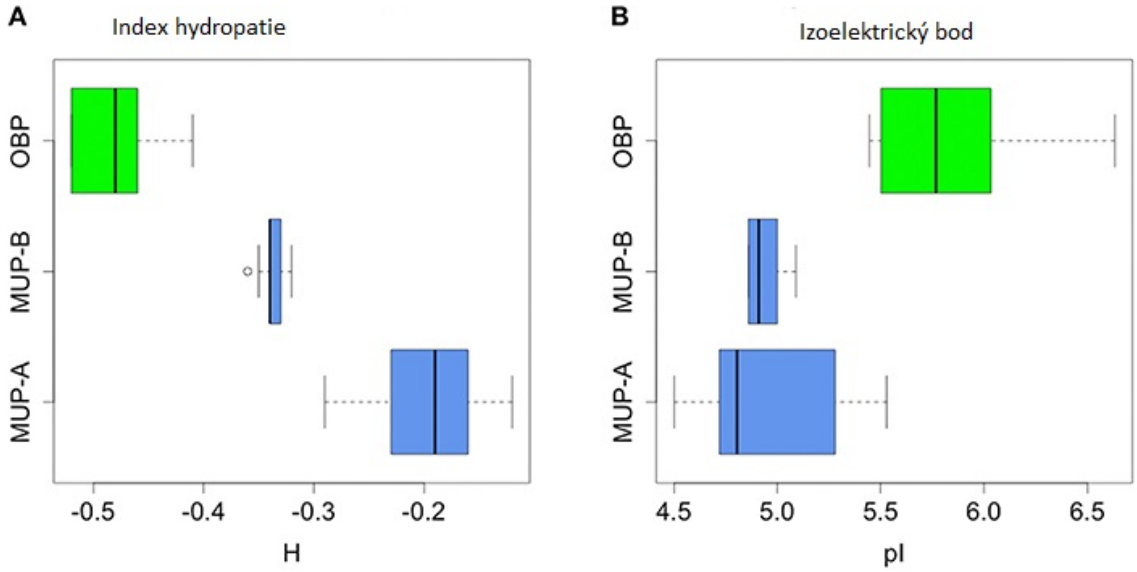
Přesná funkce jednotlivých rodin lipokalinů je zatím nejasná a je velmi pravděpodobné, že se ve svých rolích vzájemně doplňují. Zatímco LCNs se uplatňují zejména v imunitě, na chemické komunikaci se u myší z lipokalinů nejvíc podílí MUPs a OBPs. *Obp* geny jsou mezi savci časté, exprimuje je řada druhů, např. dikobrazi (*Hystrix cristata*) (Felicoli et al., 1993), sloni (*Elephas maximus*) (Lazar et al., 2002), prase (*Sus scrofa*) (Spinelli et al., 1998), kráva (*Bos taurus*) (Bignetti et al., 1985), potkan (*Rattus norvegicus*) a norník rudý (*Myodes glareolus*) (Stopková et al., 2010). U křečků (*Cricetus cricetus*) je znám OBP homologní protein Afrodisin z vaginálního sekretu samic (Briand et al., 2000a). Potkani (*Rattus norvegicus*) mají jeden jediný pravý *Obp*, pojmenovaný *Obp1f* resp. *Obp1* (Briand et al., 2000b). Ostatní názvy označující *Obp* geny potkana jsou ve skutečnosti ortology jiných lipokalinů. *Obp2b* je homologní k myšímu *Lcn14* a *Obp3* je alpha-2u-globulin, tudíž má blíže k myším *Mup.*

Mup geny nejsou u savců na rozdíl od *Obp* časté, hojně zastoupené jsou pouze u myší a potkanů, zřídka jsou přítomny u jiných druhů ve více kopiích. U potkanů existují k myším *Mups* homologní alpha-2u-globuliny, hlavní exkreční proteiny v moči samců (Roy et al., 1966) čítající osm zástupců (anotace v Ensembl databázi). Lipokalin se schopností vazby feromonálních ligandů, tzv. SAL, je znám i ze

submaxilárních žláz u kance. Fylogeneticky je blízký MUPs (Marchese et al., 1998; Spinelli et al., 2002).

Na základě odlišných biochemických vlastností tj. indexu hydropatie (H) a izoelektrického bodu (pI) lze usoudit, že myší MUPs a OBPs jsou ve svých ligandech a funkcích komplementární. Vzhledem k větší hydrofobicitě váží MUPs spíše více nepolární ligandy než OBPs a současně obě rodiny fungují za odlišného pH prostředí. OBPs by tak mohly svou afinitou k méně hydrofóbním ligandům být předurčeny k vyvazování nespecifických produktů degradace (Stopková et al., 2014; Stopková et al., 2016).

Obr.3. Komplementární vlastnosti MUPs a OBPs (převzato z Stopková et al., 2016)



MUPs mají vyšší hydrofobicitu a současně nižší izoelektrický bod než OBPs.

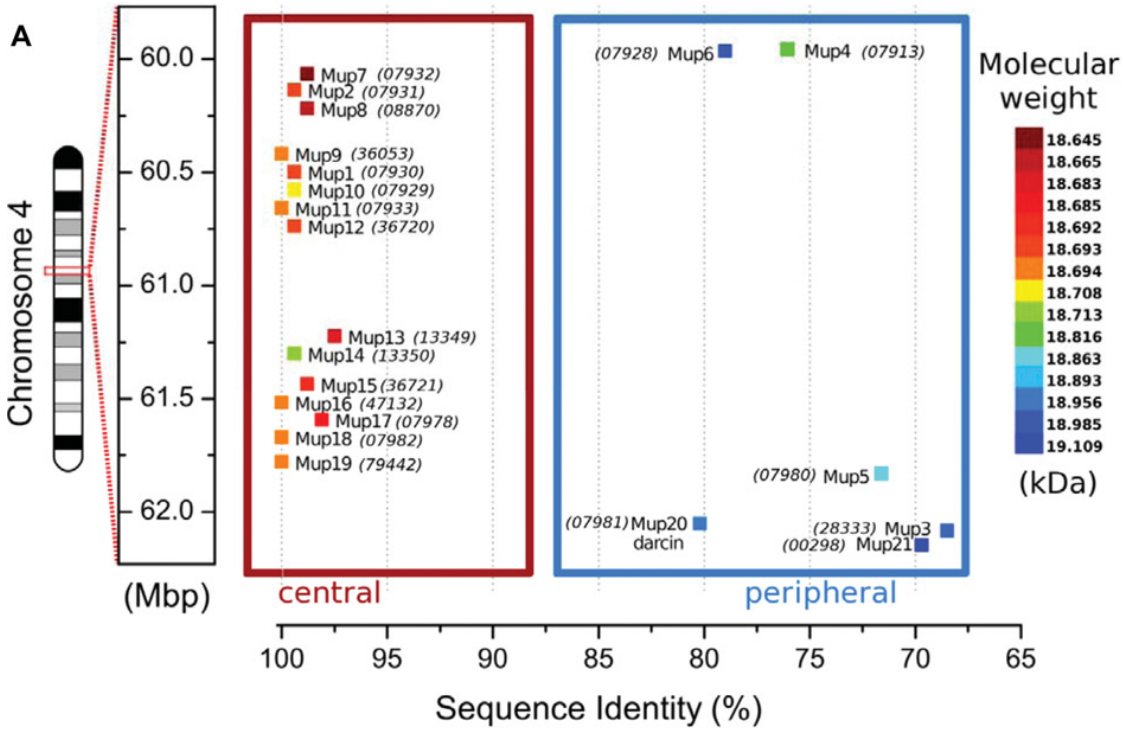
3.3 Hlavní močové proteiny (Major urinary proteins - MUPs)

MUPs jsou jednoznačně nejvíc studovanou skupinou lipokalinů podílející se na chemické komunikaci. Je to i díky jejich početnosti a produkovaném množství u modelového organismu - myši (*Mus musculus*). Hlavním expresním orgánem MUPs jsou játra, odtud se filtrací krve skrze ledviny dostávají močí do externího prostředí. Myší moč obsahuje v porovnání s jinými druhy savců fyziologicky velké množství bílkovin a je zde výrazný pohlavní dimorfismus v abundanci močových proteinů. U samců je to až 70 mg/ml, u samic 3-4x méně, přičemž až 99% tvoří právě MUPs (Humphries et al., 1999). Kromě jater jsou MUPs exprimovány v dalších tkáních jako je např. slinná žláza, MOE/VNO, NALT (angl. nasal-associated lymphoid tissue) a lakrimální žlázy (Shahan et al., 1987; Utsumi et al., 1999; Stopková et al., 2016;). Jejich funkce zde je však zatím nejasná.

Geny *Mup*s prodělaly v minulosti u hlodavců duplikaci. U myší vytváří klastr na chromozomu 4, který zahrnuje 21 funkčních genů a 21 pseudogenů. Označení genů je v databázích velmi chaotické, protože existují dvě verze pojmenování, a to buď dle Logan et al., 2008, nebo označení z MGI. Například Loganův *Mup1* je *Mup4* dle MGI. V této práci se řídím nomenklaturou z MGI.

Funkční geny jsou podle sekvenční podobnosti rozděleny na dvě skupiny. Skupina A (group-A *Mup*s) obsahuje *Mup*s z okrajových oblastí klastru, v některých publikacích jsou označovány též jako "periferní" *Mup*s. Vykazují nízkou sekvenční podobnost, 67-81% na úrovní aminokyselinové sekvence, a patří sem 6 zástupců (*Mup3, Mup4, Mup5, Mup6, Mup20/darcin, Mup21*). Ostatních 15 *Mup*s patří do skupiny B, tvz. centrální *Mup*s (group-B *Mup*s), s více než 97% cDNA identitou. Tyto Mups jsou velmi obtížně identifikovatelné, liší se i jednou jedinou aminokyselinou a na úrovni proteinu je dokonce několik identických (Logan et al., 2008; Phelan et al., 2014b). Sekvenční odlišnosti mezi A a B *Mup*s se odrážejí i ve struktuře, zejména v šířce barelu. B-*Mup*s mají barel široký, otevřený pro vazbu objemných ligandů, naopak zúžený barel A-*Mup*s vykazuje vyšší selektivitu a afinitu pro malé těkavé molekuly (Phelan et al., 2014b).

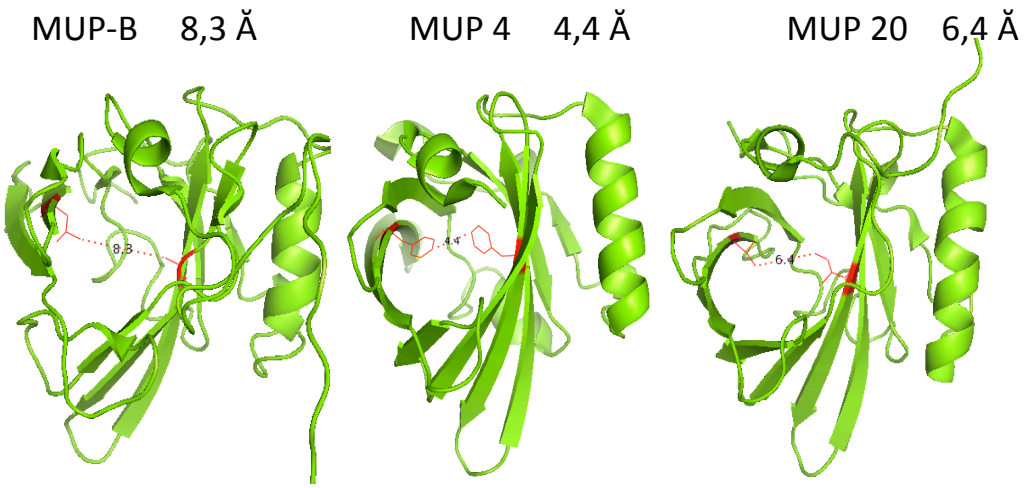
Obr.4. Organizace *Mup*s genů na chromozomu 4 (převzato z Phelan et al., 2014b)



Zvětšená oblast B3Q chromozomu 4 s očíslovanými páry bazí. Rozdělení *Mup*s genů na centrální (červený rámeček) a periferní (modrý rámeček) podle molekulární hmotnosti maturovaných proteinů jasně odráží rozdíly v sekvencích mezi oběma skupinami (centrální 97% identita x periferní 67-91% identita) MUPs.

Exprese MUPs je positivně ovlivněna testosteronem, tyroxinem a růstovým hormonem (Hastie et al., 1979; Knopf et al.,1983). Uměle podávaný testosteron juvenilům, kastrovaným samcům i samicím vyrovná expresi MUPs na úroveň u dospělého samce, a to jak kvantitativně, tak kvalitativně (Hastie et al., 1979; Clissold et al., 1984). U samic byla zjištěna fluktuace hladiny MUPs v průběhu estrálního cyklu s maximem na začátku estru. Samice tak mohou využít potenciál MUPs k signalizaci své perceptivity (Stopka et al., 2007).

Obr.5. Porovnání šířky kavity centrálních a periferních MUPs



Vzdálenost v Ă měřena mezi ekvivalentními aminokyselinami. Vytvořeno v programu PyMOL s využitím PDB struktur (2LB6, 3KFF, 2L9C).

Za primární funkci MUPs se považuje export feromonů do vnějšího prostředí. Těkavé feromony jsou ve vodě nestabilní a velmi rychle degradují. Vazbou do kavity MUPs získají ligandy ochranu a navíc se zpomalí jejich uvolňování z pachových značek (Hurst et al., 1998), které jsou tak pro okolí déle detekovatelné. Pachové značky informují případné sexuální partnery, či naopak kompetitory, o dominanci a teritoriu (Hurst, 1993; Janotova & Stopka, 2011; Nelson et al., 2015). Samci tyto značky ve svém teritoriu neustále obnovují a vzájemně si je se svými "soupeři" v hraničních oblastech přeznačkovávají (Humphries et al., 1999). Míra značkování zároveň positivně koreluje s úspěchem samce při reprodukci (Thonhauser et al., 2013).

Močovým MUPs byla také přisuzována funkce v rozpoznávání individuality jedince, tedy k odlišení příbuzných i možného sexuálního partnera. Podle tzv. "barcode hypothesis" a MUPs polymorfismu má mít každý jedinec odlišný profil těchto proteinů v moči, unikátní kód individuality (Hurst et al., 2001). Ten je dán kombinacemi různých variací v aminokyselinách v kódujících oblastech a odlišnou transkripcí centrálních MUPs mezi jedinci (Sheehan et al., 2016). Thoß et al. ve své studii však polymorfismus MUPs v myší populaci (*M. m. musculus*) popírají a dokonce ukazují na nestabilitu MUPs profilu v průběhu puberty a dospělosti (Thoß et al., 2015; 2016).

MUPs kromě feromonů mohou vázat i xenobiotika, podobně jako OBPs (Grolli et al., 2006). Příkladem u jaterních MUPs je potencionálně toxický 2,4-di-terc-butylfenol, který nahradil u testované myši přirozený ligand MUPs v moči, feromon 2-sec-butyl-4,5-dihydrothiazol (Kwak et al., 2016). MUPs by tak mohly fungovat (společně s OBPs a LCNs) jako sběrači toxických produktů, např. ROS.

Funkce detoxifikace organismu a transport ligandů ven z těla je dle navržené hypotézy "Toxic waste hypothesis of chemical communication" (Stopková et al., 2009) primární (ancestrální) rolí lipokalinů, ze které byla později odvozena funkce těchto ligandů v chemické komunikaci. Dle této teorie chemická komunikace vznikla jako evoluční přizpůsobení čichu na toxické organické metabolity a především pak na ty, které korelují s tělesnou a reprodukční zdatností individua.

3.3.1 MUPs, močové feromony a účinky na fyziologii a chování

Sexuálně dimorfní exprese jaterních MUPs nesoucích těkavé feromonální ligandy dává samcům možnost ovlivňovat reprodukční fyziologii a chování samic a zároveň tyto molekuly hrají roli v interakcích mezi samci. I samice mají v moči svůj specifický feromon. Feromony mohou svým působením na neuroendokrinní systém vyvolat řadu efektů v závislosti na pohlaví, stáří a dominanci recipientního zvířete. Jeden feromon může vyvolat více efektů a současně určité chování či fyziologická změna může být zapříčiněna působením různých feromonů.

Akcelerace nástupu puberty u samic, tzv. Vandenbergh efekt (Vandenbergh, 1969; Vandenbergh et al. 1975) je příklad efektu vyvolaného působením více feromonů. Jsou to 2-sec-4,5-butyl-dihydrothiazol (SBT), 3,4-dehydro-exo-brevikomin (DHB), 6-hydroxy-6-metyl-3-heptanon (HMH) a α- a ß-farneseny (produkty prepuciálních žláz). Současně se ovšem SBT, DBH a farneseny uplatňují i v tzv. Whitten efektu, tj. při synchronizaci a prodloužení estru samic a indukci ovulace (Whitten et al., 1968; Jemiolo et al., 1986), a navíc působí na samice jako atraktanty (Jemiolo et al., 1985). U samců DHB a SBT vyvolávají vzájemnou agresi (Novotný et al., 1985).

Přerušení gravidity samic, tzv. Bruce efekt (Bruce, 1959) je vyvolán přítomností cizího samce či pouze jeho moči. Efektory, které aborci indukují, nejsou známy, ale pravděpodobně se jedná o nízkomolekulární frakci, tj. těkavé komponenty moči (Peele

et al., 2003). Jako další atraktant samic byl identifikován (metylthio)metanetiol (MTMT), který je přítomen pouze v moči samců (Zhang et al., 2005).

Zajímavý je samičí feromon, 2,5-dimetylpyrazin (DMP), který je uvolňovaný u samic chovaných skupinově. DMP oddaluje u juvenilních samic i samců nástup puberty (Jemiolo and Novotný, 1994) a zároveň potlačuje estrus tzv. Lee-Boot efekt (Ma et al., 1998). Tento feromon tak může negativně ovlivňovat populační densitu divoce žijících myší. U samců DMP negativně ovlivňuje spermatogenezi. U testovaných laboratorních CBA samců po expozici DMP vzrostla frekvence abnormalit na hlavičkách spermií vzniklých v důsledku poškození DNA v průběhu meiózy (Daev & Dukelskaya, 2003).

Role darcinu jako feromonu je ovšem poměrně sporná vzhledem k několika okolnostem. Darcin je charakterizován u poddruhu *Mus musculus domesticus* jako samčí specifický (male-specific) MUP sekretovaný močí, který má roli v určování dominance a při teritoriální kompetici a vzájemné agresivitě samců (Nelson et al., 2015). V působení na samice je pak darcinu přisuzována role atraktantu v době estru. Stimuluje u nich učení a paměť specifické pachové stopy konkrétního samce, tudíž samice pro páření nepreferuje pach jiného (Roberts et al., 2010). Navíc míra jeho exprese informuje okolí o fitness samce, protože u oslabených jedinců množství MUP20 v moči klesá (Lopes & Konig 2016). Ovšem v našem výzkumu prováděném na poddruhu *M. m. musculus* darcin rozhodně není unikátní pro samce a je exprimován i jinde než v játrech - byl detekován ve slinách obou pohlaví (Stopka et al., 2016), v proteomu slz (Stopková et al., 2017) a dokonce i v oviduktu, děložním sekretu (Yip et al., 2013) a vaginálním sekretu samic (Černá et al., 2017). Zde exprese darcinu narůstá v době přechodu proestru v estrus, kdy je hladina na nejvyšší úrovni a přetrvává do metestru (Černá et al., 2017). Z toho vyplývá, že údajný samčí feromon je produkovaný i samicemi, a to především v receptivní fázi estrálního cyklu. Je proto těžké si představit, že by tento protein sám o sobě stimuloval specifické odpovědi u samic. Pravděpodobnější je, že významnou roli hraje ligand/směs ligandů vynášených tímto proteinem (v kombinaci s jinými lipokaliny), např. SBT, ke kterému má darcin vysokou afinitu (Phelan et al., 2014a). Moč je plná těkavých ligandů a je tudíž velmi malá pravděpodobnost, že při vysoké afinitě darcinu k SBT by zůstal tento protein „prázdný“ a působil sám o sobě.

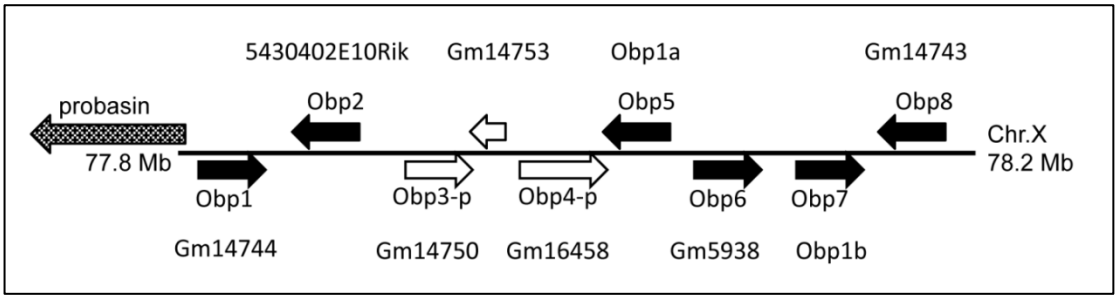
3.4 Odoranty vázající proteiny (Odorant binding proteins - OBPs)

OBPs představují další klastr proteinů, který se u myší významně podílí na chemické komunikaci. První objevený OBP byl vzhledem k svému ligandu, 2-isobutyl-3-metoxypyrazinu, pojmenován jako pyrazin-vázající protein (angl. pyrazin-binding protein - PBP). Byl izolován z čichové sliznice krav (Bignetti et al., 1985). Struktura kravského OBP je atypická lipokalinů, tvoří ji pohyblivý dimer, přičemž vzniká další vazebné místo pro ligand na jeho rozhraní v kontaktu s okolním rozpouštědlem, a neobsahuje charakteristické cysteiny pro tvorbu disulfidické vazby. Prasečí OBP je monomer, klasický lipokalin s disulfidickou vazbou (Spinelli et al., 1998), který ovšem díky post-translační modifikaci, O-glykosylaci, vytváří isoformy. O-glykosylace probíhá extracelulárně a předpokládanou funkcí je ovlivnění vazebné specifity ligandů (Nagnan-Le Meillour et al., 2014). Potkaní OBP je taktéž monomer se schopností vazby pyrazinu. I přes odlišnou strukturu má řada OBP různých savců některé ligandy společné, např. pyrazin, thymol, undecanal (Monte et al., 1993).

U myši společně s probasinem vytváří geny pro *Obp* monofyletickou skupinu lokalizovanou na chromozomu X. Krystalografická struktura žádného z myších OBP ještě nebyla zjištěna, ale dle sekvenční podobnosti k potkanímu OBP1F lze přepokládat monomerní uspořádání. Tak jako *Mups*, *Obp* geny prodělaly sérii duplikací a tvoří klastr 6 genů a 2 pseudogenů (Stopková et al., 2014). *Obp* geny lokalizované na X chromozomu mimo klasických strukturních motivů lipokalinů obsahují navíc C-X-X-X-C motiv. Tento motiv obsahuje i *Afrodisin*, homolog myších *Obp* exprimovaný ve vagíně samic křečků (Briand et al., 2000a), potkaní *Obp1f* (Briand et al., 2000b) a většina *Obp* genů u různých druhů savců analyzovaných z dostupných databází (Ensembl).

.

Obr.6. Organizace *Obp* genů na chromozomu X (převzato ze Stopková et al., 2014)



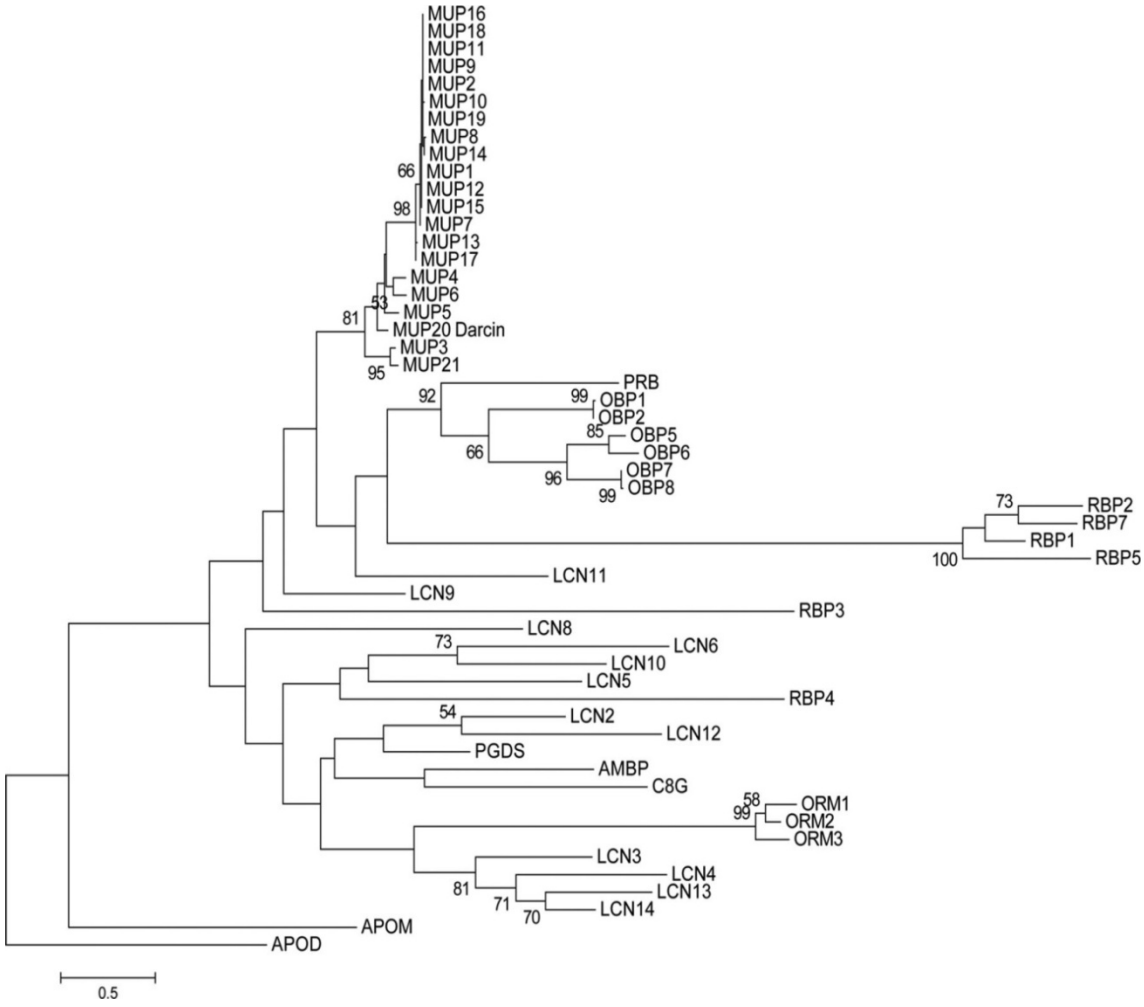
Pozice Obp genů na X chromozomu očíslovaných podle pořadí (včetně Ensembl kódů), 5 genů (černé šipky) a 2 pseudogeny (bílé šipky). Pseudogen Gm14753 nepatří mezi Obp, je to retroelement.

OBP je přisuzována řada funkcí. Samozřejmě jednou z nich je pasivní transport hydrofóbních ligandů k jejich receptorům, čímž je ochraňují před hydrofilním prostředí sliznice (Pevsner & Snyder 1990). Škála ligandů, které jsou OBP schopné vázat je velmi široká, např. kravský OBP v laboratorních podmínkách vázal na 80 ligandů s různou molekulární hmotností a chemickými vlastnostmi (Pevsner et al., 1990).

Přímá interakce OBPs (či komplexu OBP/ligand) s receptorem nebyla nikdy potvrzena a účast na transdukci signálu je zatím nepodložená. OBPs mohou naopak sloužit k rychlému odstraňování ligandů/odorantů z blízkosti čichových receptorů a pomáhat tak k jejich senzibilaci a čištění mukózy při vysoké koncentraci ligandu. Jedním z příkladů může být výzkum provedený na rekombinantním, fluorescenčně značeném OBP5 (původně OBP1a). Rekombinantní OBP5 s navázaným ligandem je internalizováno do čichové sliznice skrze megalinový receptor (LRP2) v mikroklcích podpůrných buněk. Pouze komplexy OBP5/ligand jsou přes LRP včleněny do lysosomu, samotné OBP5 nikoliv (Strotmann & Breer, 2011). Tímto způsobem mohou být odoranty velmi rychle odstraněny z dosahu receptorů. Další příklad přiklánějící se k funkci OBP jako scavengeru odorantů podává Lazar et al. Ve své studii sloního OBP poukazuje na příliš pomalou vazbu a následnou disociaci komplexu OBP/ligand pro evokování změny v chování (zde je ligandem pre-ovulatorní feromon v moči samic iniciující kopulační chování) a vylučuje tím fungování OBP jakožto přenašeče feromonů k receptorům (Lazar et al., 2002).

Díky schopnosti vazby konečného, reaktivního produktu peroxidace lipidů, 4-hydroxy-2-nonenalu (HNE), se předpokladá i role vychytávače (angl. scavenger) toxických molekul a ochrana sliznic (Grolli et al., 2006).

Obr.7. Dendrogram myších lipokalinů (převzato z Stopková et al., 2014)



Evoluční historie lipokalinů s ApoD jako ancestrálním předkem. Čísla u větví odpovídají procentu stromů, ve kterých při výpočtech asociované taxy klastrovaly dohromady. Použita metoda maximum-likelihood založená na JTT matrix-based modelu, program MEGA5.

3.5 Další významné proteiny chemické komunikace

Lipokaliny nejsou jediná proteinová rodina, která se podílí na chemické komunikaci. Ač k tomu mají díky své terciární struktuře s ligand-vázajícím barelem nejlepší předpoklady, i jednoduché proteiny typu exokrinních sekrečních peptidů (angl. exocrine gland-secreted peptides - ESPs) se do chemické komunikace zapojují

a to tak, že samotné fungují jako signál. Další velmi obsáhlá rodina proteinů, sekretoglobiny (angl. secretoglobins - SCGBs) vážou ligandy odlišné od těch u MUPs a OBPs a rozšiřují tak repertoár potenciálních chemosignálů.

3.5.1 Exocrine gland-secreted peptides (ESPs)

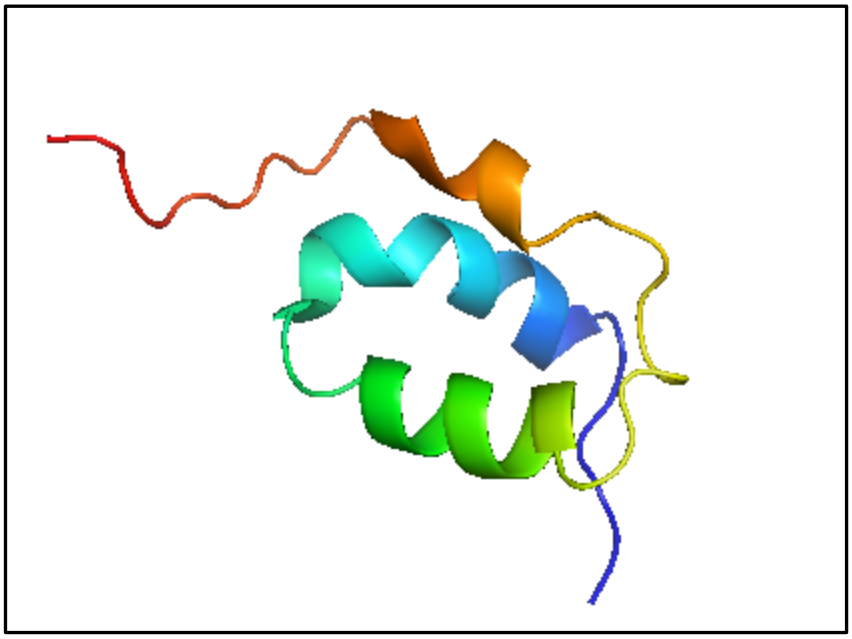
Rodina ESPs je u myší lokalizována na 17. chromozomu a je tvořena 38 geny, z čehož 14 tvoří pseudogeny. Peptidy mají variabilní délku od 60 do 160 aminokyselin, přičemž v oblasti N-koncové domény, tvořené 10-15 aminokyselinami, vykazují nejvyšší homologii. ESPs byly popsány poměrně nedávno, v roce 2005, kdy Kimoto et al., identifikoval 7kDa peptid schopný stimulovat V2R neurony vomeronasálního orgánu. Tento peptid je produkován extraorbitálními lakrimálními žlázami samců a sekretován do slz, proto název exocrine gland-secreted peptid 1 (ESP1) (Kimoto et al., 2005). Později byl popsán i receptor pro ESP1, V2Rp5, který po aktivaci u samic stimuluje receptivní chování, lordózu v zádech pro usnadnění kopulace. Signální dráhy v mozku, které toto chování řídí, jsou pohlavně dimorfní (viz. kap. 4.4.) (Haga et al., 2010). Samice mají také svůj specifický ESP, konkrétně ESP36, jehož funkce zatím nebyla popsána. Ovšem díky pohlavnímu dimorfismu v expresi těchto dvou ESPs byla zjištěna závislost exprese na testosteronu. V jeho přítomnosti je ESP1 upregulován, kdežto ESP36 je downregulován. Zajímavé je, že v případě ESP1 výše zmíněné platí jen u juvenilů, kastrace u adultního samce nezmění hladinu ESP1 (Kimoto et al., 2007).

Další ESP s popsanou feromonální funkcí je ESP22, exprimovaný lakrimálními žlázami u juvenilů obou pohlaví myší ve věku 2-3 týdnů. Funkcí tohoto feromonu je inhibice sexuálního chování dospělých jedinců právě vůči juvenilům. Exprese ESP22 je u nich až padesátkrát vyšší než dospělců. Účinek feromonu byl ověřen na samicích v estru, kdy rekombinantní ESP22 výrazně inhiboval sexuální chování samců (Ferrero et al., 2013).

Výsledky našeho výzkumu jsou ovšem opět v rozporu s dosavadními pracemi. ESP1 jsme detekovali v proteomu slin a nosního sekretu, a přestože byla jeho exprese významně vyšší u samců, jeho přítomnost byla zaznamenána i u samic (Stopka et al., 2016; Kuntová et al., 2017). ESP22 společně s ESP1 patřily k nejvíce exprimovaným

proteinům v nosním sekretu u dospělých jedinců divokých myší (Kuntová et al., 2017).

Obr.8. Příklad 3D struktury ESP - ESP1



Vytvořeno v programu PyMol s využitím PDB struktury (2LMK) z Protein Data Bank databáze.

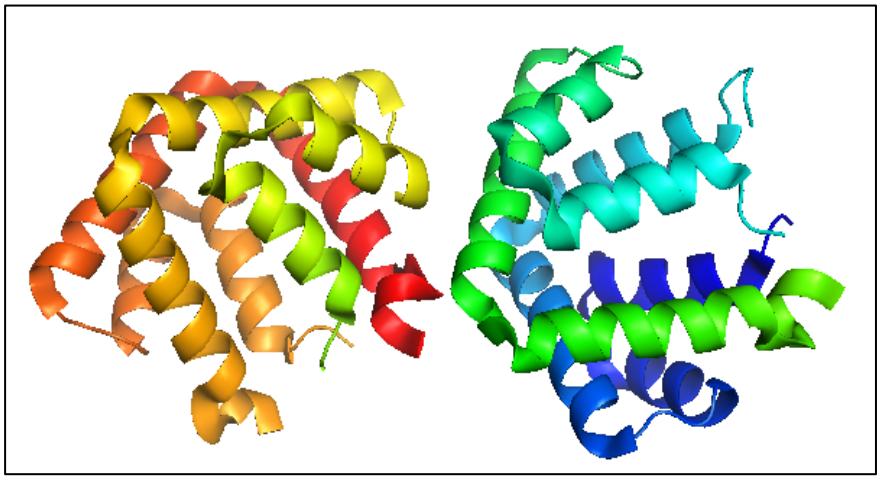
3.5.2 Sekretoglobiny (SCGBs)

Sekretoglobiny představují rodinu malých sekrečních proteinů identifikovaných zatím pouze u savců. Váží variabilní ligandy od steroidních hormonů po eikosanoidní mediátory zánětu (Beat et al., 1976; Austin et al., 2004; Mukherjee et al., 2007). Jsou složkou řady sekretů včetně těch ze slzných a slinných žláz, prostaty, dělohy a plic (Beier, 2000; Austin et al., 2004; Mukherjee et al., 2007). Jejich terciární struktura připomíná strukturu globinů, proto je v názvu přípona -globin. Funkčně vytváří dimery složené ze dvou čtyř-α-helixových monomerů s hydrofobní kapsou pro vazbu ligandů. Tato struktura je konzervativní, ovšem aminokyselinová sekvence nikoliv, podobně jako je tomu u lipokalinů (Callebaut et al., 2000).

Prvním objeveným SCBG byl uteroglobin (UG, Scgb1a1), původně pojmenovaný blastokinin, v děloze králíků z časné fáze embryogeneze, kterou stimuluje. Později se ukázalo, že je exprimován v řadě dalších tkání a byl znovu několikrát přejmenován (Clara cell 10-kDa protein - CC10, Clara cell 16 kDa protein - CC16, urine protein-1).

UG je multifunkční protein s protizánětlivými a imunomodulačními účinky (review Mukherjee et al., 2007). Další zajímavý zástupce je kočičí alergen Fel D1, který vyvolává imunitní odpověď na srst koček formou IgE protilátek. Zodpovídá za 95% alergií na kočky (Ohman et al., 1974). Z podčelistní žlázy a slin samců prasete byl izolován feromaxien, sekretoglobin vázající 16-androsten feromony, které po interakci se slinami indukují u samic estrus (Austin et al., 2004). Řada sekreteglobinů funguje v humánní medicíně jako markery, např. mammaglobin-B (Scgb2a1) je asociován s rakovinou vaječníků (Tassi et al., 2009) a lipophilin-B (Scgb1d2) s rakovinou prs (Culleton et al., 2006).

Obr.9. Příklad 3D struktury sekretoglobinu - Fel D1



Vytvořeno v programu PyMol s využitím PDB struktury (1PUO) z Protein Data Bank databáze.

U myši existuje 68 genů pro Scgb lokalizovaných na různých chromozomech (zejména chr. 7). 64 paralogů z těchto Scgb patří do rodiny tzv. androgen vázajících proteinů (angl. Androgen binding proteins - ABPs). ABPs jsou tvořeny heterodimery z 34 typů podjednotek SCGB1B (ABPA-like) a 30 typů SCGB2B (ABPBG-like) spojených disulfidickými můstky. Jsou exprimovány v různých tkáních orofaciální oblasti, např. slinné žlázy, VNO, MOE, slzné žlázy, příušní žláza, ale i ve vaječnících a prostatě (Laukaitis et al., 2005). U těchto proteinů se předpokládá účast na chemické komunikaci, ačkoliv například u experimentálních jedinců dvou poddruhů *Mus musculus* ABP signál neindukoval zvýšenou potřebu očichávání, jako je tomu u močových značek, tudíž zde neexistovala preference pro žádný z poddruhů (Bímová et al., 2009). To znamená, že ABPs se neuplatňují ve vzájemném rozeznávání

poddruhů. V dalším případě byl zkoumán efekt knock-outu genů *Abpa27* a *Abpbg27* u laboratorního kmene myši. Výsledkem byla preference slinných značek opačného pohlaví, které obsahovaly oba ABPs (Chung et al. 2017). Myši tedy jsou schopné ABPs ze slin cítit, ovšem jejich skutečná role v chemické komunikaci musí být ještě prověřena.

4 POHLAVNÍ DIMORFISMUS V TKÁŇOVÉ EXPRESI LIPOKALINŮ A V SIGNÁLNÍ TRANSDUKCI

Samice i samci vykazují repertoár chování, které je pro každé pohlaví specifické. Každé toto chování je primárně evokováno jako odpověď na specifický podnět některého ze senzorů. Takovým stimulem může být zbarvení, námluvní píseň a tanec, či právě chemický signál.

Velká část chemosensorického systému u myší funguje u obou pohlaví stejně. Je to ta část, která zajišťuje základní biologické potřeby jako vyhledání potravy a bezpečí. Minoritní část, sexuálně dimorfní, odlišuje obě pohlaví specifitou vnímaných ligandů, expresí transportních proteinů, transdukčními dráhami v mozku a ve finále i výsledným projevem formou pohlavně-specifického chování.

Ve výsledcích našich a i jiných skupin (Ibarra-Soria et al., 2014) se ukazuje, že na úrovni receptorů mezi pohlavími nejsou významné rozdíly a že dimorfismus tudíž musí být zajišťován jinými mechanismy, především na úrovni signálu. Lipokaliny, jakožto přenašeče signálu, mají pravděpodobně na dimorfismu v olfakci svůj podíl. Představují totiž hojně exprimované geny/proteiny různých tkání orofaciální oblasti a ačkoliv jejich funkce nejsou stále ještě přesně specifikované, právě pohlavní dimorfismus v expresi by je mohl pomoci objasnit.

V následujících podkapitolách sumarizuji dostupné publikace o pohlavním dimorfismu v olfakci u myší, především z pohledu odlišné tkáňové exprese lipokalinů a dalších významných genů/proteinů chemické komunikace, ale také na úrovni následné transdukce signálu.

4.1 Tkáně nosní dutiny (VNO, MOE, NALT) a proteom nosního sekretu

V dřívějších publikacích byla exprese lipokalinů ve tkáních často popisována u jednoho či několika zástupců. S vědeckým pokrokem a zpřístupněním metody sekvenování se rozšířilo spektrum lipokalinů o řadu nových zástupců. Bylo tak umožněno detekovat i minimální rozdíly v sekvencích a přesně charakterizovat jednotlivé proteiny a jejich tkáňovou specifitu.

Přítomnost MUPs (MUP4 a MUP5) a OBPs v nosní tkáni u myši byla potvrzena metodou *in situ* hybridizace (Utsumi et al., 1999). Metodou sekvenování mRNA byl popsán transkriptom MOE u laboratorního kmenu myši BALB/c (Shiao et al., 2012). Výsledkem byla detekce 1088 genů pro olfaktorické receptory (*Olfr*), z čehož 254 genů mělo vyšší hladinu exprese u samců (angl. male-biased) než u samic. Obecně je hladina exprese jednotlivých *Olfr* nízká vzhledem k fungování detekce odorantů formou jeden neuron - jeden receptor. Rozdílný výsledek byl zaznamenán u genů *Obp*s, které měly vyšší expresi u samic (angl. female-biased), ačkoliv u obou pohlaví je hladina exprese v porovnání s *Olfr* vysoká. Konkrétními detekovanými *Obp*s byly (popsáno v naší nomenklatuře Stopková et al., 2016) *Obp1, Obp2, Obp5, Obp7* a *Obp8*. Taktéž detekované geny pro *Lcns* - *Lcn11, Lcn13* a *Lcn14* byly v této publikaci více exprimované u samic. Z genů pro *Mup*s byly detekovány pouze dva - *Mup4* a *Mup5*, jejichž exprese byla popsána jako stejná u obou pohlaví (Shiao et al., 2012).

Podobná analýza transkriptomu MOE, a navíc VNO, u kmene C57BL/6 se zaměřením na receptory přinesla naprosto odlišné výsledky. Výzkum prováděný taktéž metodou sekvenování mRNA objevil pouze minimální pohlavní dimorfismus na úrovni *Olfr* a vomeronasální receptorů (*Vmnr*). Co se lipokalinů týče, popisují zde vysokou variabilitu v expresi mezi jedinci, konkrétně samci. V transkriptomu MOE a VNO detekovali souhrně mRNA 13 lipokalinů, konkrétně *Mup4*, *Mup5*, *Obp1*, *Obp2*, *Obp5*, *Obp6*, *Obp7*, *Lcn2*, *Lcn3, Lcn4*, *Lcn11*, *Lcn13* a *Lcn14*, plus nově charakterizovali *Lcn16* a *Lcn17* jako vomeronasální transkripty. Na rozdíl od předchozí publikace nebyl v MOE potvrzen žádný pohlavní dimorfismus lipokalinů, přestože *Obp*s a *Mup*s patřily k vysoce exprimovaným genům této tkáně. Ve VNO naopak pohlavní dimorfismus lipokalinů zaznamenán byl, exprese *Mup*s a *Obp*s byla signifikantně male-biased. Geny pro *Lcn*s nevykazují v této publikaci žádný pohlavní dimorfismus (Ibarra-Soria et al., 2014).

V naší publikaci srovnávající tkáňovou expresi lipokalinů mezi dvěma poddruhy myši (*M. m. musculus* a *M. m. domesticus*) jsme charakterizovali hladinu vybraných lipokalinů pomocí qPCR (Real-Time PCR). Potvrdili jsme expresi odorant binding proteinů, které jsme před tím detekovali pouze bioinformaticky (Stopková et al., 2014), konkrétně *Obp1*, *Obp5*, *Obp7*, dále pak *Mup4*, *Mup5*, *Lcn11* a VNO-specifický *Lcn4*. Exprese *Obp*s v nosních tkáních (VNO, MOE, NALT) byla vysoká, nicméně statisticky nevykazovala signifikantní rozdíl mezi pohlavími ani mezi poddruhy myší. Nově identifikované *Obp*s se od *Mup*s liší především tím, že jejich beta barel je méně hydrofobní a tudíž spektrum ligandů, které *Obp*s může transportovat je pravděpodobně odlišné od ligandů, které nosí *Mup*s (Stopková et al., 2016).

Mnohem detailnější výsledky přinesla naše analýza využívající sekvenování celého transkriptomu nosních tkání (VNO a MOE) a nLC-MS/MS detekce proteinů (Kuntová et al., 2017). I tato práce potvrdila již předchozí výsledky naše i dalších autorů, že pohlavní dimorfismus v detekci signálu (nosní tkáni) na úrovni transkriptomu je minimální a ve VNO ani v MOE nebyl prokázán žádný významný pohlavní dimorfismus na úrovni exprese. Z celkového počtu cca 25 500 genových transkriptů VNO a MOE je pouze 10 dimorfních. Většinou jde o geny pohlavních chromozomů. Nedetekovali jsme ani žádný pohlavní dimorfismus v expresi lipokalinů. Co se abundance týče, lipokaliny, společně s antimikrobiálními proteiny, patří mezi nejhojnější transkripty. Ve VNO i MOE je exprimován *Obp1, Obp2, Obp3-ps* (pseudogen), *Obp5, Obp7, Obp8, Mup4, Mup5, Lcn2, Lcn3, Lcn4, Lcn11, Lcn13* a *Lcn14*. V MOE je navíc exprimováno *Obp6*, *Mup6* a dokonce *Mup-ps22* (pseudogen). Je tedy zřejmé, že lipokaliny v nosní tkáni jsou důležité pro obě pohlaví.

Na úrovni proteomu (sekret nosní dutiny) jsme detekovali mnohem větší pohlavní dimorfismus. Z celkového počtu 21 typů lipokalinů se jako dimorfní potvrdila exprese group-B MUPs, jejichž přítomnost v nose v dosavadních publikacích popsána nebyla. Opačný trend byl zaznamenán u LCN11, jež byl exprimován více u samic. Z dalších proteinů chemické komunikace jsme detekovali ESP1. Na rozdíl od výsledků ze slz nebyl v nosním sekretu unikátní pro samce, byl zaznamenán v menší míře i u samic. Spolu s ESP1 patřil i SCGB2B20 (taktéž male-biased) k nejčetnějším proteinům v nosním sekretu samců. Na úrovni transkriptomu však tyto geny detekovány nebyly, což svědčí o možnosti, že tyto produkty mohou pocházet se slz (Kuntová et al., 2017).

4.2 Lakrimální žlázy a proteom slz

Na rozdíl od *Obp*s, exprese *Mup*s v lakrimálních žlázách myší byla prokázána již dříve (Shahan et al., 1987). Objev pohlavního dimorfismu u ESP1 a jeho účinků (Kimoto et al., 2005) pak poukázal na možnou signální funkci slz. Je tedy možné, že pohlavně dimorfní genová exprese lakrimálních žláz je součástí sexuální signalizace a je významným zdrojem proteinů účastnících se chemické komunikace.

V naší primární publikaci zaměřené na expresi lipokalinů v různých tkáních byla právě lakrimální žláza (angl. lacrimal gland - LG) místem s největším pohlavním dimorfismem lipokalinů. Detekovali jsme zde většinu analyzovaných lipokalinů, konkrétně *Obp5, Obp6, Obp7, Lcn11, Mup4* a *Mup5*, přičemž *Obp7* byl vychýlený ve prospěch samic a *Mup4* naopak ve prospěch samců, a to u obou testovaných poddruhů myši (Stopková et al., 2016).

Pro získání přesnějších informací jsme v následující publikaci (Stopková et al. 2017) provedli analýzu proteomu myších slz a sekvenaci mRNA exorbitálních lakrimálních žláz. Na úrovni proteomu jsme detekovali OBP1, OBP5, OBP6, OBP7, MUP4, MUP5, MUP10, MUP20, MUP17/13 (nelze specifikovat vzhledem k velké podobnosti MUPs skupiny B), LCN11 a LCN13 plus řadu proteinů z rodiny sekretoglobinů (např. SCGB1B19, SCGB1B3, SCGB2A2-Mammaglobin, SCGB2B3, SCGB2B7) a ESPs (např. ESP1, ESP6, ESP18, ESP38). Z transkriptomu koreluje s proteomem exprese *Obp5, Obp7* (oba female-biased), *Mup4* (male-biased) a několik ESPs a SCGBs. Odchylky mezi zastoupením transkriptů vs. proteinů mohou být dány expresí v jiných, s produkcí slz asociovaných, tkáních, např. na úrovni proteinu hojně zastoupené OBP1, je pravděpodobně exprimováno jinými žlázami, např. infra-orbitální žlázy, přídatné lakrimální žlázy či epiteliální buňky oční mukózy.

Lipokaliny, konkrétně OBP1, OBP5, OBP7 a LCN11 patří dokonce mezi nejpočetnější proteiny slz. OBP7 je jediný female-biased lipokalin, male-biased lipokalin je MUP4. Dalšími proteiny, jejichž exprese převažovala u samců, bylo několik zástupců z rodiny sekretoglobinů (SCGB1B19, SCGB1B3, SCGB2A2 - Mammaglobin, SCGB2B3 a SCGB2B7) a ESP38. Naše výsledky potvrdily i výsledky dřívější publikace (Kimoto et al., 2005), která popisuje protein ESP1 jako specifický samčí feromon, neboť jsme ho detekovali pouze u samců (Stopková et al., 2017).

4.3 Slinné žlázy a proteom slin

Po detekci lipokalinů v submandibulární slinné žláze (angl. submandibullary gland - SMG) v naší prvotní publikaci, v níž jsme využili pouze 10 vybraných genů, se pomocí qPCR ukázalo, že tato žláza neexprimuje žádný z *Obp*s ani *Lcn*s a jediným detekovaným lipokalinem byl pouze *Mup5* (Stopková et al., 2016). Cílem další publikace bylo proto identifikovat kompletní proteom slin a zjistit, zda se nějaké lipokaliny vyskytují ve slinách, přestože nejsou exprimovány hlavní slinnou žlázou SMG (Stopka et al., 2016).

Na úrovni sekvenace transkriptomu SMG jsme potvrdili nepřítomnost mRNA pro *Obp* geny. Zároveň byla v transkriptomu detekována exprese dalších variant *Mup*s, konkrétně *Mup20/darcin,* a to překvapivě u obou pohlaví (v jiných publikacích uváděn jako specifický samčí feromon z moči), *Mup4, Mup5* a *Mup9*, což odpovídá detekci *Mup*s v slinných žlázách v dřívějších pracích (Shahan et al., 1987).

Proteom slin naopak, v porovnání s transkriptomem, vykazuje nezvykle velkou variabilitu a je na lipokaliny velmi bohatý. Detekovali jsme celkem 20 z 55 lipokalinů ze skupin LCNs (LCN2, LCN3, LCN4, LCN11, LCN12, LCN13, LCN14), z OBPs (OBP1, OBP2, OBP5, OBP6 a OBP7) a MUPs (MUP4, MUP5, MUP6, MUP8, MUP14, MUP17, MUP20, MUP21). Z vyjmenovaných lipokalinů bylo 10 pohlavně dimorfních, a to 9 ve prospěch samce (OBP1, OBP2, LCN3, LCN4, LCN13, LCN14, MUP4, MUP14 a MUP20) a pouze MUP8 byl exprimován více u samic. Z dalších proteinů chemické signalizace byl ve slinách přítomen ESP1 a ESP6, a přestože množství těchto genů bylo vyšší u samců, určitá hladina exprese byla zaznamenána i u samic. To přináší otázku, zda může ESP1 (případně MUP20/darcin – viz výše) fungovat jako specifický samčí signál, když je v dalších tkáních produkovaný i samicemi. Rodina sekretoglobinů byla ve slinách také hojně zastoupena, někteří zástupci byli ekvivalentně exprimováni u obou pohlaví, v případě pohlavního dimorfismu převažovala vždy exprese na straně samců (např. SCGB1B2, SCGB2B2, SCGB1B27). Z celkového proteomu je tedy zřejmé, že lipokaliny jsou významnou součástí slin, které mohou být během selfgroomingu (čištění srsti) deponovány do srsti a sloužit tak jako zdroj chemických signálů.

4.4 Pohlavní dimorfismus v transdukci chemického signálu

Jeden chemický signál, feromon, je schopný u každého pohlaví vyvolat jinou reakci, ať už v chování či ve změně fyziologického stavu. Příkladem může být již zmiňovaný 2-sec-butyl-4,5-dihydrothiazol, který u samic ovlivňuje estrus a samců iniciuje vzájemnou agresi. Pohlavní dimorfismus v olfaktorické signalizaci a následné "odpovědi" může být teoreticky na úrovni transportérů, sensorických receptorů, ale i v projekcích mozku. Receptivitu neuronů vůči chemosignálům navíc může ovlivňovat i fyziologický stav.

Na úrovni receptorů VNO i MOE nebyl u myší detekován výrazný pohlavní dimorfismus, který by měl účinky na pohlavně-specifické chování či fyziologii (Ibarra-Soria et al., 2014; Kuntová et al., 2017). Významný podíl na sex-specifickém chování má Trpc2 kanál ve VSNs a feromonální ligandy VNO, které jej ovlivňují (Kimchi et al., 2007). Reprimují tak samčí (univerzální nastavení u obou pohlaví) a aktivují samičí chování myší (viz kapitola 2.3.).

Na příkladu ESP1 byl detekován dimorfismus až na úrovni neurální aktivity v mozku. Po expozici ESP1 se u obou pohlaví zapíná *c-Fos* exprese v odlišných částech, a to u samic ve ventromediálním hypotalamu, kdežto u samců v mediální preoptické oblasti. To může vést k odlišnostem v pohlavně specifickém chování evokovaném feromonem (Haga et al., 2010). Ačkoliv pravděpodobně existuje i jiný mechanismus, protože u samců jiného laboratorního kmene nedocházelo k indukci exprese *c-Fos* ve VNO po expozici ESP1 vůbec (Kimoto et al., 2005). Ti samci, kteří exprimovali ESP1 ve svých slzách, nejsou responzivní vůči exogennímu ESP1, samci, kteří jej neexprimují (Haga et al., 2010), responzivní jsou. Tento rozdíl může být způsoben tím, že receptory pro ESP1 jsou u exprimujících samců zahlceny vlastním proteinem a responsibilita na exogenní ESP1 tak selhává kvůli desensitizaci receptorů vůči ligandu. Rozpor přinášejí i naše výsledky z proteomu nosního sekretu a slin, kde je ESP1 přítomen i u samic (Stopka et al., 2016; Kuntová et al., 2017). Podobně ESP22, charakterizovaný jako protein juvenilů, jsme detekovali v proteomu nosního sekretu dospělých samců i samic (Kuntová et al., 2017).

Velkou roli v senzibilitě neuronů na feromony mají i hormony. Chování samic se během cyklu dramaticky mění a chemosignál, který v receptivním období estru funguje jako atraktant, v diestru nikoliv. Takovým signálem jsou feromony MUPs a vše je dáno odlišnou citlivostí senzorických neuronů v každém období cyklu. MUP-

responsivní neurony VNO jsou v době diestru umlčovány progesteronem. Exprimují totiž unikátní elementy signální transdukce, které jsou inaktivovány fosforylací iniciovanou proteinem PGRMC1 (progesterone receptor membrane component1 protein). Progesteron tak přímo působí na VSNs a inhibuje jejich schopnost detekovat MUPs (Dey et al., 2015).

5 PŘÍSPĚVEK AUTORKY

Tato disertační práce je zaměřena na lipokaliny v chemické komunikaci, zejména na úrovni olfakce, a jejich dimorfismus v expresi v různých tkáních orofaciální oblasti. Nejlépe prostudovanou skupinou lipokalinů, která se do chemické komunikace zapojuje, jsou játry exprimované MUPs sekretované do moči. Zde je jejich funkce transportérů feromonálních signálů již dobře známá, a stejně tak účinky jejich nativních ligandů na fyziologii a chování příslušníků druhu. Odlišná je situace u lipokalinů exprimovaných v orofaciální oblasti. Publikace zahrnuté v mé disertační práci pomáhají odhalit expresní místa různých rodin lipokalinů (MUPs, OBPs, LCNs) a dalších proteinů chemické komunikace (SCGBs a ESPs) v tkáních orofaciální oblasti. V publikacích, které jsou součástí mé disertační práce, jsme se zaměřili na studium proteomu slz, nosního sekretu a slin, a na expresi žláz odpovídajících (alespoň z části) za produkci těchto sekretů. Zajímaly nás odlišnosti u pohlaví, z nichž by se dalo usuzovat na funkci lipokalinů v těchto tkáních a jejich role v sexuální komunikaci u myší.

5.1 Příloha 1

Stopková, R., **Dudková, B.**, Hájková, P., & Stopka, P. (2014).
Complementary roles of mouse lipocalins in chemical communication and immunity.
Biochem.Soc.Trans.42,893-898

Review přináší přehled známých lipokalinů (OBPs, MUPs, LCNs) a usuzuje na komplementární fungování jednotlivých rodin v rámci chemické komunikace a imunity. Na základě bioinformatické analýzy je zde předložen aktualizovaný seznam predikovaných transkriptů pro *Obp*s u myší (které jsou později ověřeny v následujících publikacích). Lipokaliny představují velmi variabilní skupinu s širokou škálou potenciálních ligandů, někteří jsou specializovaní pro učitou funkci (tj. role v imunitě nebo v exkreci chemického signálu), jiní mají multiúčelový potenciál (např. ve transportu chemosignálů a vychytání toxických molekul). Poukazujeme zde na chemické vlastnosti (index hydrofobicity, izoelektrický bod) koexprimovaných rodin

lipokalinů (OBPs, group-A a group- B MUPs), které se nepřekrývají a tudíž ve svých vlastnostech a rolích mohou být navzájem komplementární.

5.2 Příloha 2

Stopková, R., Vinkler, D., **Kuntová, B**., Šedo, O., Albrecht, T., Suchan, J., Dvořáková-Hortová, K., Zdráhal, Z.& Stopka, P. (2016).
Mouse lipocalins (MUP, OBP, LCN) are co-expressed in tissues involved in chemical communication.
Frontiers in Ecology and Evolution, *4*, 47.

Cílem této publikace bylo analyzovat expresi zástupců z rodiny lipokalinů (OBPs, MUPs, LCNs) napříč různými tkáněmi a žlázami (celkem 8 různých tkání), které se mohou podílet na chemické komunikaci. Současně jsme chtěli porovnat pohlavní dimorfismus a inter-specifické rozdíly u dvou poddruhů rodu *M. musculus* (*M. m. musculus* a *M. m. domesticus*) v expresi lipokalinů. Naše výsledky ukazují koexpresi různých druhů lipokalinů, a to především v orofaciální oblasti (VNO, MOE, NALT, LG), čímž se rozšiřuje spektrum možných vázaných ligandů. Pohlavní dimorfismus na úrovni transkriptu byl detekován pouze u dvou zástupců v LG (*Mup4* - male biased, *Obp7* - female biased), nicméně tento výsledek je stejný pro oba zkoumané poddruhy myší. Inter-specifické rozdíly byly zaznamenány pouze v celkově vyšší expresi lipokalinů ve VNO u poddruhu *M. m. musculus*.

5.3 Příloha 3

Stopka, P., **Kuntová, B**., Klempt, P., Havrdová, L., Černá, M., & Stopková, R. (2016).
On the saliva proteome of the Eastern European house mouse (*Mus musculus musculus*) focusing on sexual signalling and immunity.
Scientific Reports, *6*.

Publikace se zaměřuje na studium transkriptomu submandibulárních žláz a především pak proteomu slin. Cílem bylo ukázat potenciál slin v chemické komunikaci, a to na přítomnosti proteinů z rodiny lipokalinů. Navzdory detekované variabilitě

a abundanci lipokalinů v proteomu slin, submandibulární žlázy exprimují pouze několik zástupců (*Mup*s, nikoliv však *Obp*s či *Lcn*s). Ve slinách jsou hojně zastoupeny OBPs, MUPs a LCNs u obou pohlaví, přičemž z deseti pohlavně dimorfních je devět více exprimováno u samců. Detekovali jsme zde i přítomnost MUP20/darcin (mRNA i protein) a ESP1 (protein) u obou pohlaví, dříve charakterizované jako typicky samčí proteiny. Vzhledem k odlišnosti v poměru mRNA/protein je zřejmé, že slinné lipokaliny jsou produkovány v jiných tkáních, např. v dalších menších slinných žlázách jako jsou příušní žlázy či podjazykové žlázy. Je také možné, že produkty dalších orofaciálních žláz, zejména MOE, VNO a LG se mohou dostávat do slin skrze nasolakrimální dukt a palatální duct (*ductus incisivum*). Kromě proteinů spojených s chemickou komunikací jsme detekovali i řadu proteinů zahrnutých do procesů hojení zranění, imunitních a neimunitních odpovědí na patogeny, což dokumentuje i významnou roli slin jako ochranné bariéry.

5.4 Příloha 4

Stopková, R., Klempt, P., **Kuntová, B.**, Stopka, P. (2017).
On the tear proteome of the house mouse (*Mus musculus musculus*) in relation to chemical signalling.
PeerJ 5:e3541.

Primární funkcí slz je udržovat oční bulvu čistou a zvlhčovat její povrch. Ochraňují tak oko před vlivy z vnějšího prostředí a mají i funkci v imunitě a chemické komunikaci. V publikaci jsme analyzovali proteom slz a transkriptom lakrimálních žláz myši domácí (*M. m. musculus*) a porovnávali rozdíly v expresi lipokalinů u obou pohlaví. MUPs, OBPs a ESPs jsou v slzách hojně zastoupeny a patří mezi nejexprimovanější proteiny - z 5% nejzastoupenějších tvoří asi polovinu lipokaliny. Samice obecně exprimují více OBPs, samci více MUPs, ESPs a sekretoglobinů. V proteomu oka je ESP1 čistě samčí protein, na rozdíl od MUP20/darcin, který je přítomen u obou pohlaví. Dimorfismus jsme detekovali i v expresi baktericidních proteinů, což poukazuje na fakt, že obě pohlaví mohou ovlivňovat mikrobiotické prostředí odlišně a degradací tak produkovat odlišné těkavé ligandy.

5.5 Příloha 5

Kuntová B., Stopková R., Stopka, P. (2017).
The olfactory transcriptome and proteome of the house mouse (*Mus musculus musculus*).
V recenzním řízení.

Cílem publikace bylo analyzovat transkriptom olfaktorických tkání (MOE a VNO) a rozpustný proteom nosní dutiny u myši domácí (*M. m. musculus*). Srovnáním transkriptomů MOE a VNO u samců a samic jsme hledali rozdíly v expresi, které by mohly přispívat k pohlavně-specifické detekci feromonů. Celkový pohlavní dimorfismus v epxresi mRNA byl však velmi malý. Mezi nejhojnější transkripty u obou pohlaví ovšem patřily geny kódující lipokaliny (např. *Lcn13*, *Lcn14*, *Obp*s) a celkově jsme jich v transkriptomu MOE a VNO detekovali 20 typů. Na úrovni analýzy proteomu z nosního sekretu byl pohlavní dimorfismus podstatně vyšší, z lipokalinů byly signifikantně odlišné exprese group-B MUPs (male-biased) a LCN11 (female-biased). Z dalších proteinů chemické komunikace vykazovaly ESP1, ESP22 také vyšší expresi ve prospěch samců, nicméně tyto proteiny byly detekovány i u samic, což je v rozporu s dříve publikovanými výsledky o ESP1 jako specificky samčím feromonu. Zajímavým zjištěním byla velká individuální variabilita v expresi některých proteinů, především právě z rodiny lipokalinů a některých antimikrobiálních proteinů. Pomocí korelačních matic jsme ukázali, že existují určité skupiny lipokalinů a antimikrobiálních proteinů vykazující pozitivně korelovanou míru individuální variability. To může podporovat naši původní hypotézu, že různé degradační produkty mikrobiomu, na kterém se podílí antimikrobiální proteiny, jsou následně transportovány lipokaliny, ať už na povrch těla nebo k následné degradaci v trávicím traktu.

6 ZÁVĚR

Ve své disertační práci jsem se věnovala expresi vybraných skupin lipokalinů (MUPs, OBPs, LCNs) v orofaciálních tkáních myši domácí (*M. m. musculus*). Současně jsem analyzovala míru pohlavního dimorfismu v expresi těchto proteinů na úrovni transkriptomu i proteomu, s cílem poukázat na možné působení těchto proteinů v utváření pohlavně-specifického sexuálního chování u myší.

Z celkového výčtu lipokalinů, ať už na úrovni mRNA či proteinu, je zřejmé, že jsou v orofaciální oblasti hojně zastoupené. Rozdílné výsledky transkriptomu a proteomu (tj. konkrétních orofaciálních žláz a jejich produktů) mohou být způsobeny kontinuálním tokem proteinů z místa exprese do ostatních tkání. Proteiny exprimované např. v MOE, VNO či lakrimálních žlázách se *via* nasolakrimální dukt dostávají do nosní a následně i ústní dutiny. Například lipokaliny LCN3, LCN4, LCN13 a LCN14 jsou exprimovány nosními a vomeronasálními tkáněmi, ale detekovány byly hojně i v proteomu slin. Dalším příkladem jsou ve slinách četně zastoupené OBPs, ale submandibulární žláza neexprimuje žádný. Je také možné, že na produkci orofaciálních sekretů se podílejí ještě další minoritní žlázy, které nebyly doposud analyzovány (např. infra-orbitální žlázy, příušní žláza, podjazykové žlázy, von Ebnerovy žlázy atd.).

Kromě moči jsou lipokaliny nejvíce exprimovány v nosní tkání, tvoří až 46% z celkového množství proteinů. Na úrovni transkriptomu ve všech studovaných tkáních byl pohlavní dimorfismus exprimovaných genů vždy menší nebo téměř žádný (VNO, MOE). Nicméně na úrovni proteinů prokazatelný byl a zahrnoval větší či menší počet zástupců lipokalinů. Nejvyšší počet dimorfně exprimovaných lipokalinů byl zaznamenán ve slinách. Ve srovnání s tím byl pohlavní dimorfismus lipokalinů v slzách a v nosním sekretu o něco nižší.

Překvapivě jsme zde detekovali i několik proteinů chemické komunikace, dříve charakterizovaných jako typicky samčí, u obou pohlaví. MUP20/darcin, označovaný jako močový MUP, je v našich výsledcích přítomen v proteomu slin a slz obou pohlaví. Podobně ESP1, protein lakrimálních žláz s popsanou výraznou funkcí v sexuálním chování samic, je však v proteomu nosního sekretu a slin taktéž u obou pohlaví. Poprvé jsme také detekovali zástupce MUPs ze skupiny group-B jinde než v moči, a to ve všech typech analyzovaných sekretů.

Zajímavým výsledkem je potvrzení exprese velké míry antimikrobiálních proteinů v těchto tkáních, jejichž množství a pohlavní dimorfismus vykazují podobný profil jako u lipokalinů. V poslední publikaci jsme ukázali, že interindividuální variabilita určitých skupin antimikrobiálních proteinů pozitivně koreluje s expresí různých klastrů lipokalinů a je tedy možné, že tyto skupiny proteinů mohou být součástí společného procesu degradace a přesunu degradačních produktů bakterií.

Výsledkem jsou čtyři publikace a jedna předložená k recenznímu řízení.

7 LITERATURA

Beato, M. (1976). Binding of steroids to uteroglobin. *Journal of steroid biochemistry*, *7*(5), 327-334.

Beier, H. M. (2000). The discovery of uteroglobin and its significance for reproductive biology and endocrinology. *Annals of the New York Academy of Sciences*, *923*(1), 9-24.

Bignetti, E., Cavaggioni, A., Pelosi, P., Persaud, K. C., Sorbi, R. T., & Tirindelli, R. (1985). Purification and characterisation of an odorant‐binding protein from cow nasal tissue. *European journal of biochemistry*, *149*(2), 227-231.

Bímová, B., Albrecht, T., Macholán, M., & Piálek, J. (2009). Signalling components of the house mouse mate recognition system. *Behavioural processes*, *80*(1), 20-27.

Bishop, R. E., Penfold, S. S., Frost, L. S., Höltje, J. V., & Weiner, J. H. (1995). Stationary Phase Expression of a Novel Escherichia coli Outer Membrane Lipoprotein and Its Relationship with Mammalian Apolipoprotein D IMPLICATIONS FOR THE ORIGIN OF LIPOCALINS. *Journal of Biological Chemistry*, *270*(39), 23097-23103.

Blomhoff, R., Green, M. H., Berg, T., & Norum, K. R. (1990). Transport and storage of vitamin A. *Science*, *250*(4979), 399-404.

Boehm U., Zou, Z., & Buck, L. B. (2005). Feedback loops link odor and pheromone signaling with reproduction. *Cell*, *123*(4), 683-695.

Brechbühl, J., Klaey, M., & Broillet, M. C. (2008). Grueneberg ganglion cells mediate alarm pheromone detection in mice. *Science*, *321*(5892), 1092-1095.

Briand, L., Huet, J. C., Perez, V., Lenoir, G., Nespoulous, C., Boucher, Y., & Pernollet, J. C. (2000a). Odorant and pheromone binding by aphrodisin, a hamster aphrodisiac protein. *FEBS letters*, *476*(3), 179-185.

Briand, L., Nespoulous, C., Perez, V., Rémy, J. J., Huet, J. C., & Pernollet, J. C. (2000b). Ligand‐binding properties and structural characterization of a novel rat odorant‐binding protein variant. *European Journal of Biochemistry*, *267*(10), 3079-3089.

Bruce, H. M. (1959). An exteroceptive block to pregnancy in the mouse. *Nature*, *184*(4680), 105-105.

Buck, L., & Axel, R. (1991). A novel multigene family may encode odorant receptors: a molecular basis for odor recognition. *Cell*, *65*(1), 175-187.

Callebaut, I., Poupon, A., Bally, R., Demaret, J. P., Housset, D., Delettr É, J. & Mornon, J. P. (2000). The uteroglobin fold. *Annals of the New York Academy of Sciences*, *923*(1), 90-112.

Chakraborty, S., Kaur, S., Tong, Z., Batra, S. K., & Guha, S. (2011). Neutrophil gelatinase associated lipocalin: structure, function and role in human pathogenesis. In *Acute Phase Proteins-Regulation and Functions of Acute Phase Proteins*. InTech.

Chamero, P., Marton, T. F., Logan, D. W., Flanagan, K., Cruz, J. R., Saghatelian, A., & Stowers, L. (2007). Identification of protein pheromones that promote aggressive behaviour. *Nature*, *450*(7171), 899-902.

Charron, J. B. F., Ouellet, F., Pelletier, M., Danyluk, J., Chauve, C., & Sarhan, F. (2005). Identification, expression, and evolutionary analyses of plant lipocalins. *Plant Physiology*, *139*(4), 2017-2028.

Cho, K. W., Zhou, Y., Sheng, L., & Rui, L. (2011). Lipocalin-13 regulates glucose metabolism by both insulin-dependent and insulin-independent mechanisms. *Molecular and cellular biology*, *31*(3), 450-457.

Chung, A. G., Belone, P. M., Bímová, B. V., Karn, R. C., & Laukaitis, C. M. (2017). Studies of an Androgen-Binding Protein Knockout Corroborate a Role for Salivary ABP in Mouse Communication. *Genetics*, *205*(4), 1517-1527.

Clissold, P. M., Hainey, S., & Bishop, J. O. (1984). Messenger RNAs coding for mouse major urinary proteins are differentially induced by testosterone. *Biochemical genetics*, *22*(3), 379-387.

Crossett, B., Allen, W. R., & Stewart, F. (1996). A 19 kDa protein secreted by the endometrium of the mare is a novel member of the lipocalin family. *Biochemical Journal*, *320*(1), 137-143.

Culleton, J., O'Brien, N., Ryan, B. M., Hill, A. D., McDermott, E., O'Higgins, N., & Duffy, M. J. (2007). Lipophilin B: A gene preferentially expressed in breast tissue and upregulated in breast cancer. *International journal of cancer*, *120*(5), 1087-1092.

Daev, E. V., & Dukelskaya, A. V. (2003). The female pheromone 2, 5-dimethylpyrazine induces sperm-head abnormalities in male CBA mice. *Russian Journal of Genetics*, *39*(7), 811-815.

Dey, S., Chamero, P., Pru, J. K., Chien, M. S., Ibarra-Soria, X., Spencer, K. R., ... & Stowers, L. (2015). Cyclic regulation of sensory perception by a female hormone alters behavior. *Cell*, *161*(6), 1334-1344.

Dulac, C., & Axel, R. (1995). A novel family of genes encoding putative pheromone receptors in mammals. *Cell*, *83*(2), 195-206.

Felicioli, A., Ganni, M., Garibotti, M., & Pelosi, P. (1993). Multiple types and forms of odorant-binding proteins in the old-world porcupine Hystrix cristata. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry*, *105*(3), 775-784.

Ferrero, D. M., Moeller, L. M., Osakada, T., Horio, N., Li, Q., Roy, D. S., & Liberles, S. D. (2013). A juvenile mouse pheromone inhibits sexual behaviour through the vomeronasal system. *Nature*, *502*(7471), 368-371.

Fleischer, J., Schwarzenbacher, K., & Breer, H. (2007). Expression of trace amine–associated receptors in the Grueneberg ganglion. *Chemical senses*, *32*(6), 623-631.

Fleischer, J., Schwarzenbacher, K., Besser, S., Hass, N., & Breer, H. (2006). Olfactory receptors and signalling elements in the Grueneberg ganglion. *Journal of neurochemistry*, *98*(2), 543-554.

Flo, T. H., Smith, K. D., Sato, S., Rodriguez, D. J., Holmes, M. A., Strong, R. K., & Aderem, A. (2004). Lipocalin 2 mediates an innate immune response to bacterial infection by sequestrating iron. *Nature*, *432*(7019), 917-921.

Flower, D. R., North, A. C., & Attwood, T. K. (1993). Structure and sequence relationships in the lipocalins and related proteins. *Protein Science*, *2*(5), 753-761.

Flower, D. R., North, A. C., & Sansom, C. E. (2000). The lipocalin protein family: structural and sequence overview. *Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology*, *1482*(1), 9-24.

Fluckinger, M., Haas, H., Merschak, P., Glasgow, B. J., & Redl, B. (2004). Human tear lipocalin exhibits antimicrobial activity by scavenging microbial siderophores. *Antimicrobial agents and chemotherapy*, *48*(9), 3367-3372.

Fukuhara, A., Yamada, M., Fujimori, K., Miyamoto, Y., Kusumoto, T., Nakajima, H., & Inui, T. (2012). Lipocalin-type prostaglandin D synthase protects against oxidative stress-induced neuronal cell death. *Biochemical Journal*, *443*(1), 75-84.

Fülle, H. J., Vassar, R., Foster, D. C., Yang, R. B., Axel, R., & Garbers, D. L. (1995). A receptor guanylyl cyclase expressed specifically in olfactory sensory neurons. *Proceedings of the National Academy of Sciences*, *92*(8), 3571-3575.

Ganfornina, M. D., Do Carmo, S., Lora, J. M., Torres‐Schumann, S., Vogel, M., Allhorn, M., & Sanchez, D. (2008). Apolipoprotein D is involved in the mechanisms regulating protection from oxidative stress. *Aging cell*, *7*(4), 506-515.

Grolli, S., Merli, E., Conti, V., Scaltriti, E., & Ramoni, R. (2006). Odorant binding protein has the biochemical properties of a scavenger for 4‐hydroxy‐2‐nonenal in mammalian nasal mucosa. *FEBS Journal*, *273*(22), 5131-5142.

Haga, S., Hattori, T., Sato, T., Sato, K., Matsuda, S., Kobayakawa, R., & Touhara, K. (2010). The male mouse pheromone ESP1 enhances female sexual receptive behaviour through a specific vomeronasal receptor. *Nature*, *466*(7302), 118-122.

Hastie, N. D., Held, W. A., & Toole, J. J. (1979). Multiple genes coding for the androgen-regulated major urinary proteins of the mouse. *Cell*, *17*(2), 449-457.

Hayden, S., & Teeling, E. C. (2014). The molecular biology of vertebrate olfaction. *The Anatomical Record*, *297*(11), 2216-2226.

Humphries R.E, Robertson D.H.L, Beynon R.J, Hurst J.L.(1999). Unravelling the chemical basis of competitive scent marking in house mice. Anim. Behav.;58:1177–1190.

Hurst, J. L. (1993). The priming effects of urine substrate marks on interactions between male house mice, Mus musculus domesticus Schwarz & Schwarz. *Animal Behaviour*, *45*(1), 55-81.

Hurst, J. L., Robertson, D. H., Tolladay, U., & Beynon, R. J. (1998). Proteins in urine scent marks of male house mice extend the longevity of olfactory signals. *Animal behaviour*, *55*(5), 1289-1297.

Hurst, J. L., Payne, C. E., Nevison, C. M., Marie, A. D., Humphries, R. E., Robertson, D. H., & Beynon, R. J. (2001). Individual recognition in mice mediated by major urinary proteins. *Nature*, *414*(6864), 631-634.

Ibarra-Soria, X., Levitin, M. O., Saraiva, L. R., & Logan, D. W. (2014). The olfactory transcriptomes of mice. *PLoS genetics*, *10*(9), e1004593.

Janotova, K., & Stopka, P. (2011). The level of major urinary proteins is socially regulated in wild Mus musculus musculus. *Journal of chemical ecology*, *37*(6), 647-656.

Jemiolo, B., Alberts, J., Sochinski-Wiggins, S., Harvey, S., & Novotny, M. (1985). Behavioural and endocrine responses of female mice to synthetic analogues of volatile compounds in male urine. *Animal behaviour*, *33*(4), 1114-1118.

Jemiolo, B., Harvey, S., & Novotny, M. (1986). Promotion of the Whitten effect in female mice by synthetic analogs of male urinary constituents. *Proceedings of the National Academy of Sciences*, *83*(12), 4576-4579.

Jemiolo, B., & Novotny, M. (1994). Inhibition of sexual maturation in juvenile female and male mice by a chemosignal of female origin. *Physiology & behavior*, *55*(3), 519-522.

Jia, C., Goldman, G., & Halpern, M. (1997). Development of vomeronasal receptor neuron subclasses and establishment of topographic projections to the accessory olfactory bulb. *Developmental brain research*, *102*(2), 209-216.

Jia, C., & Halpern, M. (1996). Subclasses of vomeronasal receptor neurons: differential expression of G proteins (G iα2 and G oα) and segregated projections to the accessory olfactory bulb. *Brain research*, *719*(1), 117-128.

Kang, N., & Koo, J. (2012). Olfactory receptors in non-chemosensory tissues. *BMB reports*, *45*(11), 612.

Karlson, P., & Lüscher, M. (1959). 'Pheromones': a new term for a class of biologically active substances. *Nature*, *183*(4653), 55-56.

Karunadasa, D. K., Chapman, C., & Bicknell, R. J. (2006). Expression of pheromone receptor gene families during olfactory development in the mouse: expression of a V1 receptor in the main olfactory epithelium. *European Journal of Neuroscience*, *23*(10), 2563-2572.

Keen, J. N., Caceres, I., Eliopoulos, E. E., Zagalsky, P. F., & Findlay, J. B. (1991). Complete sequence and model for the A2 subunit of the carotenoid pigment complex, crustacyanin. *The FEBS Journal*, *197*(2), 407-417.

Keverne, E. B. (2004). Importance of olfactory and vomeronasal systems for male sexual function. *Physiology & behavior*, *83*(2), 177-187.

Kimchi, T., Xu, J., & Dulac, C. (2007). A functional circuit underlying male sexual behaviour in the female mouse brain. *Nature*, *448*(7157), 1009-1014.

Kimoto, H., Sato, K., Nodari, F., Haga, S., Holy, T. E., & Touhara, K. (2007). Sex-and strain-specific expression and vomeronasal activity of mouse ESP family peptides. *Current Biology*, *17*(21), 1879-1884.

Kimoto, H., Haga, S., Sato, K., & Touhara, K. (2005). Sex-specific peptides from exocrine glands stimulate mouse vomeronasal sensory neurons. *Nature*, *437*(7060), 898-901.

Knopf, J. L., Gallagher, J. F., & Held, W. A. (1983). Differential, multihormonal regulation of the mouse major urinary protein gene family in the liver. *Molecular and cellular biology*, *3*(12), 2232-2240.

Kurahashi, T., & Menini, A. (1997). Mechanism of odorant adaptation in the olfactory receptor cell. *Nature*, *385*(6618), 725.

Kwak, J., Strasser, E., Luzynski, K., Thoß, M., & Penn, D. J. (2016). Are MUPs a toxic waste disposal system?. *PloS one*, *11*(3), e0151474.

Lareyre, J. J., Mattei, M. G., Kasper, S., Newcomer, M. E., Ong, D. E., Matusik, R. J., & Orgebin-Cristº, M. C. (1997). Structure and putative function of a murine epididymal retinoic acid-binding protein. *Journal of Reproduction and Fertility Supplement*, *53*, 59-65.

Laukaitis, C. M., Dlouhy, S. R., Emes, R. D., Ponting, C. P., & Karn, R. C. (2005). Diverse spatial, temporal, and sexual expression of recently duplicated androgen-binding protein genes in Mus musculus. *BMC evolutionary biology*, *5*(1), 40.

Lazar, J., Greenwood, D. R., Rasmussen, L. E. L., & Prestwich, G. D. (2002). Molecular and functional characterization of an odorant binding protein of the Asian elephant, Elephas maximus: implications for the role of lipocalins in mammalian olfaction. *Biochemistry*, *41*(39), 11786-11794.

Leinders-Zufall,T., Lane, A. P., Puche, A. C., Ma, W., Novotny, M. V., Shipley, M. T., & Zufall, F. (2000). Ultrasensitive pheromone detection by mammalian vomeronasal neurons. *Nature*, *405*(6788), 792-796.

Leinders-Zufall, T., Brennan, P., Widmayer, P., Maul-Pavicic, A., Jäger, M., Li, X. H., Breer, H., Zufall, F. & Boehm, T. (2004). MHC class I peptides as chemosensory signals in the vomeronasal organ. *Science*, *306*(5698), 1033-1037.

Lévai, O., Feistel, T., Breer, H., & Strotmann, J. (2006). Cells in the vomeronasal organ express odorant receptors but project to the accessory olfactory bulb. *Journal of Comparative Neurology*, *498*(4), 476-490.

Leypold, B. G., Yu, C. R., Leinders-Zufall, T., Kim, M. M., Zufall, F., & Axel, R. (2002). Altered sexual and social behaviors in trp2 mutant mice. *Proceedings of the National Academy of Sciences*, *99*(9), 6376-6381.

Liberles, S. D., & Buck, L. B. (2006). A second class of chemosensory receptors in the olfactory epithelium. *Nature*, *442*(7103), 645-650.

Ligresti, G., Aplin, A. C., Dunn, B. E., Morishita, A., & Nicosia, R. F. (2012). The acute phase reactant orosomucoid-1 is a bimodal regulator of angiogenesis with time- and context-dependent inhibitory and stimulatory properties. *PloS one*, *7*(8), e41387.

Liman, E. R., Corey, D. P., & Dulac, C. (1999). TRP2: a candidate transduction channel for mammalian pheromone sensory signaling. *Proceedings of the National Academy of Sciences*, *96*(10), 5791-5796.

Logan, D. W., Marton, T. F., & Stowers, L. (2008). Species specificity in major urinary proteins by parallel evolution. *PloS one*, *3*(9), e3280.

Lopes, P. C., & König, B. (2016). Choosing a healthy mate: sexually attractive traits as reliable indicators of current disease status in house mice. *Animal Behaviour*, *111*, 119-126.

Ma, M., Grosmaitre, X., Iwema, C. L., Baker, H., Greer, C. A., & Shepherd, G. M. (2003). Olfactory signal transduction in the mouse septal organ. *Journal of Neuroscience*, *23*(1), 317-324.

Ma, W., Miao, Z., & Novotny, M. V. (1998). Role of the adrenal gland and adrenal-mediated chemosignals in suppression of estrus in the house mouse: the lee-boot effect revisited. *Biology of reproduction*, *59*(6), 1317-1320.

Marchese, S., Pes, D., Scaloni, A., Carbone, V., & Pelosi, P. (1998). Lipocalins of boar salivary glands binding odours and pheromones. *European journal of biochemistry*, *252*(3), 563-568.

Martín-Sánchez, A., McLean, L., Beynon, R. J., Hurst, J. L., Ayala, G., Lanuza, E., & Martinez-Garcia, F. (2015). From sexual attraction to maternal aggression: when pheromones change their behavioural significance. *Hormones and behavior*, *68*, 65-76.

Martini, S., Silvotti, L., Shirazi, A., Ryba, N. J., & Tirindelli, R. (2001). Co-expression of putative pheromone receptors in the sensory neurons of the vomeronasal organ. *Journal of Neuroscience*, *21*(3), 843-848.

Matsunami, H., & Buck, L. B. (1997). A multigene family encoding a diverse array of putative pheromone receptors in mammals. *Cell*, *90*(4), 775-784.

Meredith, M., & O'connell, R. J. (1979). Efferent control of stimulus access to the hamster vomeronasal organ. *The Journal of physiology*, *286*, 301.

Miyawaki, A., Matsushita, F., Ryo, Y., & Mikoshiba, K. (1994). Possible pheromone-carrier function of two lipocalin proteins in the vomeronasal organ. *The EMBO journal*, *13*(24), 5835.

Mombaerts, P., Wang, F., Dulac, C., Chao, S. K., Nemes, A., Mendelsohn, M., Edmondson, J. & Axel, R. (1996). Visualizing an olfactory sensory map. *Cell*, *87*(4), 675-686.

Monte, M. D., Centini, M., Anselmi, C., & Pelosi, P. (1993). Binding of selected odorants to bovine and porcine odorant-binding proteins. *Chemical Senses*, *18*(6), 713-721.

Mukherjee, Anil B., Zhongjian Zhang, and Beverly S. Chilton. "Uteroglobin: a steroid-inducible immunomodulatory protein that founded the Secretoglobin superfamily." *Endocrine reviews* 28.7 (2007): 707-725.

Nagnan-Le Meillour, P., Vercoutter-Edouart, A. S., Hilliou, F., Le Danvic, C., & Lévy, F. (2014). Proteomic analysis of pig (Sus scrofa) olfactory soluble proteome reveals O-linked-N-acetylglucosaminylation of secreted odorant-binding proteins. *Frontiers in endocrinology*, *5*.

Nelson, A. C., Cunningham, C. B., Ruff, J. S., & Potts, W. K. (2015). Protein pheromone expression levels predict and respond to the formation of social dominance networks. *Journal of evolutionary biology*, *28*(6), 1213-1224.

Nishimura, K., Utsumi, K., Yuhara, M., Fujitani, Y., & Iritani, A. (1989). Identification of puberty‐accelerating pheromones in male mouse urine. *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology*, *251*(3), 300-305.

Novotny, M., Harvey, S., Jemiolo, B., & Alberts, J. (1985). Synthetic pheromones that promote inter-male aggression in mice. *Proceedings of the National Academy of Sciences*, *82*(7), 2059-2061.

Ohman, J. L., Lowell, F. C., & Bloch, K. J. (1974). Allergens of mammalian origin. *The Journal of Immunology*, *113*(6), 1668-1677.

Parmentler, M., & Libert, F. (1992). Expression of members of the putative olfactory receptor gene family in mammalian germ cells. *Nature*, *355*(6359), 453.

Peele, P., Salazar, I., Mimmack, M., Keverne, E. B., & Brennan, P. A. (2003). Low molecular weight constituents of male mouse urine mediate the pregnancy block effect and convey information about the identity of the mating male. *European Journal of Neuroscience*, *18*(3), 622-628.

Pevsner, J., Hou, V., Snowman, A. M., & Snyder, S. H. (1990). Odorant-binding protein. Characterization of ligand binding. *Journal of biological chemistry*, *265*(11), 6118-6125.

Pevsner, J., & Snyder, S. H. (1990). Odorant-binding protein: odorant transport function in the vertebrate nasal epithelium. *Chemical Senses*, *15*(2), 217-222.

Phelan, M. M., McLean, L., Armstrong, S. D., Hurst, J. L., Beynon, R. J., & Lian, L. Y. (2014a). The structure, stability and pheromone binding of the male mouse protein sex pheromone darcin. *PLoS One*, *9*(10), e108415.

Phelan, M.M., McLean, L., Hurst J.L., Beynon, R.J., Lian, L. (2014b). Comparative study of the molecular variation between ´central´and ´peripheral´MUPs and significance for behavioural signalling. *Biochemical Society Transactions*, 42(4), 866-872

Provost, P. R., Marcel, Y. L., Milne, R. W., Weech, P. K., & Rassart, E. (1991). Apolipoprotein D transcription occurs specifically in nonproliferating quiescent and senescent fibroblast cultures. *FEBS letters*, *290*(1-2), 139-141.

Rasmussen, L. E. L., Lee, T. D., Roelofs, W. L., Zhang, A., & Daves Jr, G. D. (1996). Insect pheromone in elephants. *Nature*, *379*(6567), 684.

Rassart, E., Bedirian, A., Do Carmo, S., Guinard, O., Sirois, J., Terrisse, L., & Milne, R. (2000). Apolipoprotein d. *Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology*, *1482*(1), 185-198.

Restrepo, D., Arellano, J., Oliva, A. M., Schaefer, M. L., & Lin, W. (2004). Emerging views on the distinct but related roles of the main and accessory olfactory systems in responsiveness to chemosensory signals in mice. *Hormones and behavior*, *46*(3), 247-256.

Rivière, S., Challet, L., Fluegge, D., Spehr, M., & Rodriguez, I. (2009). Formyl peptide receptor-like proteins are a novel family of vomeronasal chemosensors. *Nature*, *459*(7246), 574-577.

Rodriguez, I., Feinstein, P., & Mombaerts, P. (1999). Variable patterns of axonal projections of sensory neurons in the mouse vomeronasal system. *Cell*, *97*(2), 199-208.

Rodriguez, I., Del Punta, K., Rothman, A., Ishii, T., & Mombaerts, P. (2002). Multiple new and isolated families within the mouse superfamily of V1r vomeronasal receptors. *Nature neuroscience*, *5*(2), 134-140.

Roy, A. K., Neuhaus, O. W., & Harmison, C. R. (1966). Preparation and characterization of a sex-dependent rat urinary protein. *Biochimica et Biophysica Acta (BBA)-General Subjects*, *127*(1), 72-81.

Salier, J. P. (2000). Chromosomal location, exon/intron organization and evolution of lipocalin genes. *Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology*, *1482*(1), 25-34.

Seppälä, M., Taylor, R. N., Koistinen, H., Koistinen, R., & Milgrom, E. (2002). Glycodelin: a major lipocalin protein of the reproductive axis with diverse actions in cell recognition and differentiation. *Endocrine Reviews*, *23*(4), 401-430.

Shahan, K., Denaro, M., Gilmartin, M., Shi, Y., & Derman, E. (1987). Expression of six mouse major urinary protein genes in the mammary, parotid, sublingual, submaxillary, and lachrymal glands and in the liver. *Molecular and cellular biology*, *7*(5), 1947-1954.

Sharrow, S. D., Vaughn, J. L., Žídek, L., Novotny, M. V., & Stone, M. J. (2002). Pheromone binding by polymorphic mouse major urinary proteins. *Protein Science*, *11*(9), 2247-2256.

Sheehan, M. J., Lee, V., Corbett-Detig, R., Bi, K., Beynon, R. J., Hurst, J. L., & Nachman, M. W. (2016). Selection on coding and regulatory variation maintains individuality in major urinary protein scent marks in wild mice. *PLoS Genet*, *12*(3), e1005891.

Shiao, M. S., Chang, A. Y. F., Liao, B. Y., Ching, Y. H., Lu, M. Y. J., Chen, S. M., & Li, W. H. (2012). Transcriptomes of mouse olfactory epithelium reveal sexual differences in odorant detection. *Genome biology and evolution*, *4*(5), 703-712.

Shipley, M. T., & Ennis, M. (1996). Functionalorganization of olfactory system. *Developmental Neurobiology*, *30*(1), 123-176.

Spehr, M., Gisselmann, G., Poplawski, A., Riffell, J. A., Wetzel, C. H., Zimmer, R. K., & Hatt, H. (2003). Identification of a testicular odorant receptor mediating human sperm chemotaxis. *Science*, *299*(5615), 2054-2058.

Spehr, M., Kelliher, K. R., Li, X. H., Boehm, T., Leinders-Zufall, T., & Zufall, F. (2006). Essential role of the main olfactory system in social recognition of major histocompatibility complex peptide ligands. *Journal of Neuroscience*, *26*(7), 1961-1970.

Spinelli, S., Ramoni, R., Grolli, S., Bonicel, J., Cambillau, C., & Tegoni, M. (1998). The structure of the monomeric porcine odorant binding protein sheds light on the domain swapping mechanism. *Biochemistry*, *37*(22), 7913-7918.

Spinelli, S., Vincent, F., Pelosi, P., Tegoni, M., & Cambillau, C. (2002). Boar salivary lipocalin. *The FEBS Journal*, *269*(10), 2449-2456.

Stopka, P., Janotova, K., & Heyrovsky, D. (2007). The advertisement role of major urinary proteins in mice. *Physiology & Behavior*, *91*(5), 667-670.

Stopka, P., Kuntová, B., Klempt, P., Havrdová, L., Černá, M., & Stopková, R. (2016). On the saliva proteome of the Eastern European house mouse (Mus musculus musculus) focusing on sexual signalling and immunity. *Scientific Reports*, *6*.

Stopková, R., Dudková, B., Hájková, P., & Stopka, P. (2014). Complementary roles of mouse lipocalins in chemical communication and immunity. *Biochem. Soc. Trans*.*42*,893-898

Stopková, R., Hladovcová, D., Kokavec, J., Vyoral, D., & Stopka, P. (2009). Multiple roles of secretory lipocalins (MUP, OBP) in mice. *Folia Zoologica*, *58*, 29.

Stopková, R., Vinkler, D., Kuntová, B., Šedo, O., Albrecht, T., Suchan, J. & Stopka, P. (2016). Mouse lipocalins (MUP, OBP, LCN) are co-expressed in tissues involved in chemical communication. *Frontiers in Ecology and Evolution*, *4*, 47.

Stopková, R., Zdráhal, Z., Ryba, Š., Šedo, O., Šandera, M., & Stopka, P. (2010). Novel OBP genes similar to hamster Aphrodisin in the bank vole, Myodes glareolus. *BMC genomics*, *11*(1), 45.

Stowers, L., Holy, T. E., Meister, M., Dulac, C., & Koentges, G. (2002). Loss of sex discrimination and male-male aggression in mice deficient for TRP2. *Science*, *295*(5559), 1493-1500.

Strotmann, J., & Breer, H. (2011). Internalization of odorant-binding proteins into the mouse olfactory epithelium. *Histochemistry and cell biology*, *136*(3), 357.

Suzuki, K., Lareyre, J. J., Sánchez, D., Gutierrez, G., Araki, Y., Matusik, R. J., & Orgebin-Crist, M. C. (2004). Molecular evolution of epididymal lipocalin genes localized on mouse chromosome 2. *Gene*, *339*, 49-59.

Tassi, R. A., Calza, S., Ravaggi, A., Bignotti, E., Odicino, F. E., Tognon, G., & Romani, C. (2009). Mammaglobin B is an independent prognostic marker in epithelial ovarian cancer and its expression is associated with reduced risk of disease recurrence. *BMC cancer*, *9*(1), 253.

Tegoni, M., Ramoni, R., Bignetti, E., Spinelli, S., & Cambillau, C. (1996). Domain swapping creates a third putative combining site in bovine odorant binding protein dimer. *Nature Structural & Molecular Biology*, *3*(10), 863-867.

Thoß, M., Luzynski, K. C., Ante, M., Miller, I., & Penn, D. J. (2015). Major urinary protein (MUP) profiles show dynamic changes rather than individual 'barcode'signatures. *Frontiers in ecology and evolution*, *3*.

Thoß, M., Enk, V., Yu, H., Miller, I., Luzynski, K. C., Balint, B., Penn, D. J. (2016). Diversity of major urinary proteins (MUPs) in wild house mice. *Scientific Reports*, *6*.

Timm, D. E., Baker, L. J., Mueller, H., Zidek, L., & Novotny, M. V. (2001). Structural basis of pheromone binding to mouse major urinary protein (MUP‐I). *Protein Science*, *10*(5), 997-1004.

Utsumi, M., Ohno, K., Kawasaki, Y., Tamura, M., Kubo, T., & Tohyama, M. (1999). Expression of major urinary protein genes in the nasal glands associated with general olfaction. *Journal of neurobiology*, *39*(2), 227-236.

Vandenbergh, J. G. (1969). Male odor accelerates female sexual maturation in mice. *Endocrinology*, *84*(3), 658-660.

Vandenbergh, J. G., Whitsett, J. M., & LOMBARDI, J. R. (1975). Partial isolation of a pheromone accelerating puberty in female mice. *Journal of Reproduction and Fertility*, *43*(3), 515-523.

White, C. A., Ghazan-Shahi, S., & Adams, M. A. (2015). β-Trace protein: a marker of GFR and other biological pathways. *American Journal of Kidney Diseases*, *65*(1), 131-146.

Whitten, W. K., Bronson, F. H., & Greenstein, J. A. (1968). Estrus-inducing pheromone of male mice: transport by movement of air. *Science*, *161*(3841), 584-585.

Xu, F., Schaefer, M., Kida, I., Schafer, J., Liu, N., Rothman, D. L., & Shepherd, G. M. (2005). Simultaneous activation of mouse main and accessory olfactory bulbs by odors or pheromones. *Journal of Comparative Neurology*, *489*(4), 491-500.

Zhang, X., & Firestein, S. (2002). The olfactory receptor gene superfamily of the mouse. *Nature neuroscience*, *5*(2), 124-133.

Zhang, S. Z., Block, E., & Katz, L. C. (2005). Encoding social signals in the mouse main olfactory bulb. *Nature*, *434*(7032), 470-477.

Zucchi, R., Chiellini, G., Scanlan, T. S., & Grandy, D. K. (2006). Trace amine‐associated receptors and their ligands. *British journal of pharmacology*, *149*(8), 967-978.

Zufall, F., & Leinders-Zufall, T. (2007). Mammalian pheromone sensing. *Current opinion in neurobiology*, *17*(4), 483-489.

8 PŘÍLOHY

Příloha 1

Stopková, R., **Dudková, B**., Hájková, P., & Stopka, P. (2014). Complementary roles of mouse lipocalins in chemical communication and immunity. *Biochem.Soc.Trans*. *42*,893-898.

Příloha 2

Stopková, R., Vinkler, D., **Kuntová, B**., Šedo, O., Albrecht, T., Suchan, J., Dvořáková-Hortová, K., Zdráhal, Z.& Stopka, P. (2016). Mouse lipocalins (MUP, OBP, LCN) are co-expressed in tissues involved in chemical communication. *Frontiers in Ecology and Evolution*, *4*, 47.

Příloha 3

Stopka, P., **Kuntová, B**., Klempt, P., Havrdová, L., Černá, M., & Stopková, R. (2016). On the saliva proteome of the Eastern European house mouse (*Mus musculus musculus*) focusing on sexual signalling and immunity. *Scientific reports*, *6*.

Příloha 4

Stopková, R., Klempt, P., **Kuntová, B.**, Stopka, P. (2017). On the tear proteome of the house mouse (*Mus musculus musculus*) in relation to chemical signalling. *PeerJ 5*:e3541.

Příloha 5

Kuntová B., Stopková R., Stopka, P. (2017). The olfactory transcriptome and proteome of the house mouse (*Mus musculus musculus*). V recenzním řízení.

Complementary roles of mouse lipocalins in chemical communication and immunity

Romana Stopková*, Barbora Dudková*, Petra Hájková* and Pavel Stopka*[1]
*BIOCEV group, Department of Zoology, Faculty of Science, Charles University in Prague, Prague, Czech Republic

Abstract

A primary site of infection in mammals is the nostrils, representing the gate to the brain through olfactory and vomeronasal epithelia, eyes as a direct route to the brain via the optical nerve, and oral cavity representing the main route to the digestive tract. Similarly, pheromones, odorants and tastants enter animal bodies the same way. Therefore similar evolutionary forces might have shaped the evolution of systems for recognition of pathogens and chemical signals. This might have resulted in sharing various proteins among systems of recognition and filtering to decrease potential costs of evolving and utilizing unique biochemical pathways. This has been documented previously in, for example, multipurpose and widely distributed GPCRs (G-protein-coupled receptors). The aim of the present review is to explore potential functional overlaps or complementary functions of lipocalins in the system of perception of exogenous substances to reconstruct the evolutionary forces that might have shaped their synergistic functions.

Introduction

The lipocalins are a family of small extracellular proteins (typically 20 kDa) with a tertiary signature of an eight-stranded antiparallel β-barrel often with two α-helices with one on the N-terminal and the other on the C-terminal region of the protein [1]. Another typical lipocalin feature is a four to eight exon structure (i.e. frequently having seven exons and six introns) with the fixed two to five exon size [2] and a typical signature including the amino acid sequence GXW (Gly-Xaa-Trp) at a specific location in the sequence [1]. The structure of the lipocalin β-barrel is often open at one end, allowing for the binding of various hydrophobic substances in the barrel. The lipocalin family is diverse due to a lack of sequence homology on the DNA level, but the ancestral tertiary structure is conserved over the evolutionary history in all metazoan phyla, and it is generally accepted that lipocalins appeared early in the history of life [3]. This is evident by the fact that lipocalins are abundant in both prokaryotic and eukaryotic cells. Some authors suggested that eukaryotic cells have acquired genes for lipocalins from the endosymbiotic alphaproteobacterial (Gram-negative or a common ancestor of Gram-negative and Gram-positive) ancestors of the mitochondrion [4], whereas the data of Ganfornina et al. [3] provide no support for this hypothesis. In any case, the presence of lipocalins in given phyla must have been adaptive along their evolutionary history for the ability of lipocalins to bind and transport a very wide spectrum of molecules, including various harmful metabolic end-products including ROS (reactive oxygen species). A high efficiency of lipocalins binding ROS was suggested for the wide spectrum of bacterial, plant, insect and mammalian lipocalins [5,6].

Evolutionary conservancy may be linked to the inherent ability of lipocalins to bind, protect and transport various ligands of different size and structure in the aqueous environment of body fluids [1], including fatty acids, lipids, steroids, bilins, retinol, and volatile chemical signals [7], with (for example) specific effects on the receiver's physiology [8], behaviour [9,10] and mating-related memory functions [11,12]. Lipocalins are involved in diverse biological processes of which their particular importance in chemical communication and immunity is the focus of the present review.

Mouse lipocalins

The mouse lipocalin tree using Ensembl protein sequence data acquired on 5 February 2014 is represented in Figure 1. The evolutionary history was inferred by using the maximum-likelihood method based on the JTT matrix-based model [13]. Evolutionary analyses were conducted in MEGA5 [14]. Our lipocalin analysis involved 55 amino acid sequences. We have adopted deep phylogenetic inference that *ApoD* (apolipoprotein D) is most likely to be the common ancestor of vertebrate and insect lipocalins [3], which is why we rooted the tree on this particular gene.

Our maximum-likelihood analysis demonstrates that members of the MUP (major urinary protein) group of proteins represent a monophyletic cluster of genes on chromosome 4 [15] and are numbered using MGI nomenclature. Within the *Mup* cluster, there is a subtree of the recently duplicated *Mup* genes described previously as group B genes sharing high sequence homology at the level of transcripts. Six other *Mup* genes are described as group A genes [15].

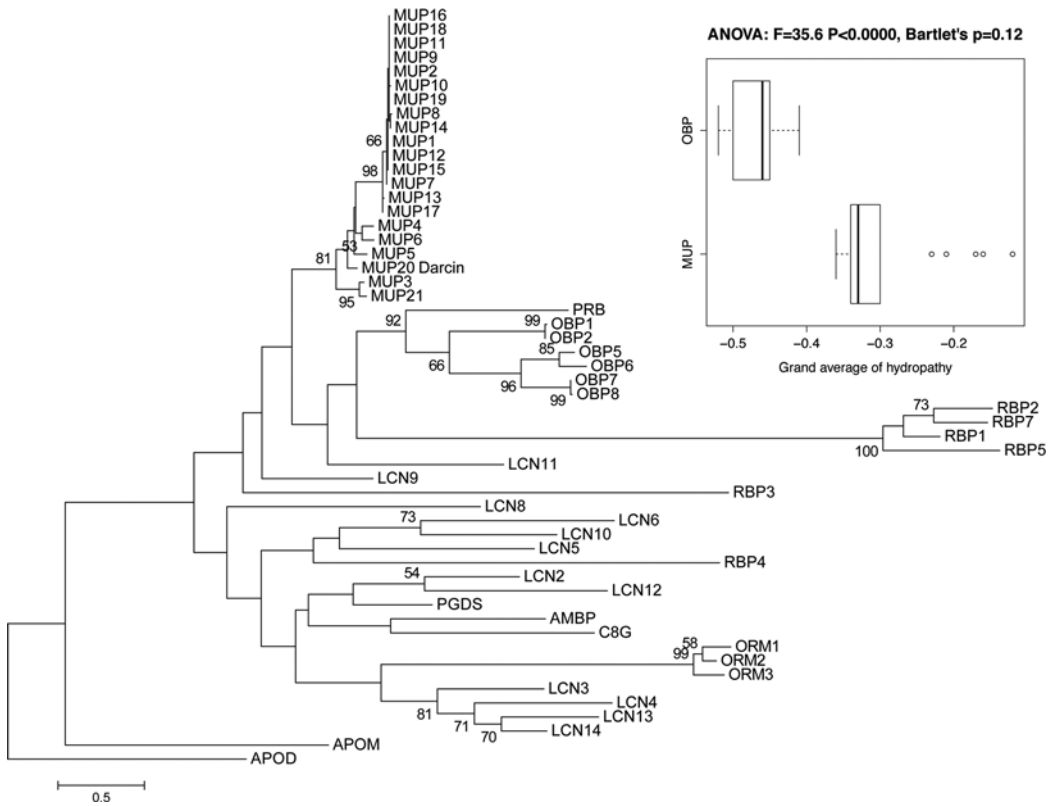
Key words: bacterium, lipocalin, major urinary protein (MUP), odorant, odorant-binding protein (OBP), pheromone.

Abbreviations: LCN, lipocalin; L-PGDS, lipocalin-type prostaglandin synthase; LPS, lipopolysaccharide; MUP, major urinary protein; OBP, odorant-binding protein; ORM, orosomucoid; PRB, probasin; ROS, reactive oxygen species.

[1] To whom correspondence should be addressed (email pstopka@natur.cuni.cz).

Figure 1 | **Dendrogram of mouse lipocalin proteins**

The evolutionary history was inferred by using the maximum-likelihood method based on the JTT matrix-based model [13]. The tree with the highest log likelihood (−9621.8856) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The initial tree for the heuristic search was obtained by applying the neighbour-joining method to a matrix of pairwise distances estimated using a JTT model. The tree is drawn to scale, with branch lengths measured as the number of substitutions per site. There were a total of 124 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 [14]. The hydrophobicity index was calculated using online resources for each member of the MUPs and OBPs with the mean and confidence intervals depicted in the inset.

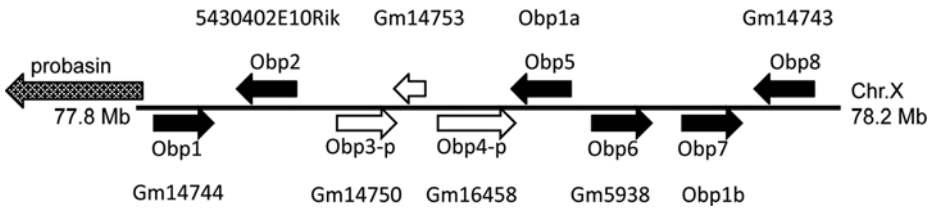


On the basis of the maximum-likelihood estimate of amino acid substitutions, the cluster of OBPs (odorant-binding proteins) along with PRB (probasin) form another monophyletic group of genes located on the mouse X chromosome. The nomenclature of *Obp* genes in Figure 2 reflects the position of *Obp1*–*Obp8* on DNA. The genomic organization of *Obp* genes is depicted in Figure 2, showing that there are at least two *Obp* pseudogenes between *Obp2* and *Obp5*. We have decided to include two *Obp*-like pseudogenes (gm14750 and gm16458) in the numbering because they seem to be untruncated and we cannot exclude the possibility that some of them are expressed in other wild muroid rodents. The six coding *Obp*s can be clearly separated into three groups: (i) *Obp1* and *Obp2* sharing 99 % similarity, (ii) *Obp5* and *Obp6*, and (iii) *Obp7* and *Obp8*. A common feature of the entire cluster is a specific disulfide bond (Cys^{38}–Cys^{42}), which represents a strong motif, CXXXC (Cys-Xaa-Xaa-Xaa-Cys) [16]. Interestingly, this motif is found only in X-linked *Obp* genes in various rodent species (i.e. rat Obp1f and hamster aphrodisin). *Obp* genes that are located on different chromosomes, do not have this motif and, moreover, some in fact cluster more strongly with other types of lipocalins instead, e.g. human *Obp2a* and *Obp2b* and rat *Obp2b* cluster with a group of *Lcn* (lipocalin) genes (R. Stopková and P. Stopka, unpublished work).

Genes for LCN2–LCN14 are located on mouse chromosome 2. The numbering was adopted from public databases (Ensembl, NCBI), but does not reflect the gene order on the chromosome. In contrast with *Mup* and *Obp* genes, *Lcn* genes are not a homogeneous gene cluster. Each member clusters with different cluster, however, with LCN3, LCN4, LCN13 and LCN14 being the most compact monophyletic group (81 bootstraps) among LCNs, and of which the expression of LCN3 and LCN4 is limited to the vomeronasal organ [17] and probably play similar roles to those of OBPs in olfactory epithelia.

Figure 2 | **Genomic organization of the mouse X-linked *Obp* genes**

Individual *Obp* members were numbered by the position on the X chromosome with the Ensembl gene codes also provided. Two pseudogenes were also included in the numbering of *Obp* members.



Pathogen-driven lipocalin evolution?

Pathogens have always been a major cause of animal mortality for their high selective impact on genomes. By developing a novel statistical framework on the basis of the correlation of allele frequencies for 500 000 SNPs in 55 distinct human populations with local environmental factors, such as diet, climate conditions and pathogen load, Fumagalli et al. [18] have been able to discern the relative contributions of environmental factors to evolution of genomes, and they found that local pathogen diversity had the strongest role in selective processes shaping the evolution of genomes. Among pathogens, bacteria play crucial roles. They are the most abundant group of organisms, and a major source of animal diseases and mortality. Bacterial cells account for the most of the Earth's biomass [19] and the 100 trillions of microbial residents of the human body outnumber human cells at a ratio of 10:1 [20]. For more than a century, living mammals were thought to have met the first bacteria at the moment of their birth. Recent studies, however, have detected bacteria even in umbilical cord blood, amniotic fluid and fetal membranes, as well as in meconium, suggesting that microbes might also be transferred to fetuses before birth (reviewed in [21]). However, most microbes are generally non-pathogenic; they exist in a fragile harmony and symbiotically with their hosts.

Nutritional immunity

Harmonious symbiosis and potential risk of exogenous bacterial infection is tightly regulated and/or reduced by a strategy called 'nutritional immunity', in part by preventing pathogens from acquiring iron [21]. During infection, bacteria rely on the host iron. It is an essential nutrient, but only small amounts of free iron are accessible, therefore bacteria acquire iron by secretion of high-affinity iron-sequestering molecules called siderophores. The mammalian host, however, limits this process by the production of LCN2 [23], which efficiently scavenges for catecholate-type siderophores (i.e. such as enterochelin, mycobactin) [24]. The interaction is driven by ionic strength between positively charged amino acid residues of LCN2 and negatively charged siderophores. The enterochelin-type siderophore-binding pocket of LCN2 is evolutionarily conserved, thus it represents an efficient defence mainly against Gram-negative bacterial species such as *Escherichia coli*, *Klebsiella pneumoniae*, *Brucella abortus* and *Salmonella* spp. [24,25]. Altered anti-inflammatory functions of LCN2 have been demonstrated on *Lcn2*$^{-/-}$ mice infected with sublethal doses of *E. coli.* Furthermore, the normal phenotype was restored when enterochelin was added exogenously [24]. Functions that are complementary to those that are evident for LCN2 evolved in human and rat tear lipocalin LCN1 [26] and in mouse LCN12 [27]. LCN1 is constantly present in tears, nasal mucus and tracheal secretions [26]. LCN1 also scavenges for siderophores but, in contrast with LCN2, LCN1 primarily binds bacterial and fungal hydroxymate-type siderophores [28]. Furthermore, LCN1 represents a general scavenger of potentially harmful lipophilic molecules, such as products of lipid peroxidation [29], on the retinal surfaces of the eye. Another lipocalin, L-PGDS (lipocalin-type prostaglandin synthase) protects various cells including neuronal cells by scavenging ROS, a common product of bacterial degradation [30]. Another role for L-PGDS has been discovered by artificial administration of bacterial endotoxin LPS (lipopolysaccharide) into lungs. LPS is recognized by Toll-like receptor 4 and up-regulates the production of L-PGDS, which in turn stimulates the production of prostaglandin 2 that facilitates the recruitment of neutrophils and removal of pathogens [31].

Orosomucoid lipocalins (ORM1–ORM3) seem to be a true scaffold of an immune system for their capacity to inhibit neutrophil chemotaxis and superoxide production, lymphocyte proliferation, and platelet aggregation when an animal is injured (reviewed in [32]). ORM1 is primarily produced by mural cells and is an important regulator of the angiogenic response to injury [32]. Furthermore, the level of orosomucoids in the blood rises during infectious diseases culminating during acute phase of infection [33].

MUP members have a capacity to bind volatile ligands due to an increased number of hydrophobic residua in their binding pocket. However, the role of several members of MUPs may go beyond their traditionally described functions. Urinary MUPs are produced by the liver where they may also play roles specific to liver metabolism. Such a dual function stemmed out of the study by Petrak et al. [34] that provided evidence for the nutritional content to balance the expression of several members of the MUP family in the liver (i.e. mice overloaded with iron up-regulate *Mup* genes). However, such

MUP increase may also be explained by common regulation within metabolic pathways which leaves this hypothesis open until it is tested further.

Many lipocalins are produced by nasal tissues. In MOE (major olfactory epithelia) and AOE (accessory olfactory epithelia), MUPs as well as OBPs are present [35] and may also scavenge for iron to prevent bacterial growth similarly as has been demonstrated for LCN2 and LCN1 [22]. Other research published to date, however, suggests that their newly evolved roles are more important.

Lipocalins with hydrophobic pockets are involved in chemical communication

Mouse MUPs were subject to hundreds of studies and there is prevailing agreement that they are involved in the protection and transport of volatile ligands relevant to chemosignalling. They are mainly synthesized in the liver, excreted in urine in large quantities [36,37] and are believed to play important roles in individual recognition [38]. Species-specificity of chemical signals (e.g. *Mus musculus musculus* and *Mus musculus domesticus*) is believed to be manifested through differential expression of *Mup* genes such that the level of their sexual dimorphism is species-specific [39]. The cost of chemosignalling is optimized by social regulation whereby males significantly increase MUP production when caged with females behind a metal grid in inbred (C57Bl6) [40] as well as in wild (*M. musculus musculus*) [41] mice. Similarly, female MUP production is presumably regulated by sex hormones (i.e. potentially by oestradiol) because MUP production continuously follows the oestrous cycle, with the highest level reached just 1 day before ovulation, a period called behavioural oestrus [39]. Despite their high sequence homology, MUPs are involved in causing various specific effects upon the receiver's reproductive physiology (reviewed in [16,42], and elsewhere in this issue of *Biochemical Society Transactions*) and individual recognition-related memory function [11,12].

The most enigmatic lipocalins are members of mouse X-linked OBPs. The first mammalian OBP was isolated from cow nasal mucosa and has been shown to bind the specific 'green-smelling compound' 2-isobutyl-3-methoxypyrazine, which is why the first described *Obp* gene was named pyrazine-binding protein [43]. Pevsner at al. [44] later shown that OBPs interact with a broad range of ligands of different structure and are found across different mammalian taxa [16]. For example, hamster aphrodisin is a true OBP family member, also possessing the CXXXC sequence motif typical of X-linked mouse OBPs and PRB, evidently binds volatile pheromones [45] and significantly clusters within the mouse *Obp5–Obp8* subtree similarly to the bank vole (*Myodes glareolus*) *Obp* genes [46]. In addition, hamster *Msp* (male-specific salivary protein) and *Flp* (female specific lacrimal protein) also belong to an *Obp* group due to their high sequence similarity and having the CXXXC motif (reviewed in [16]).

The involvement of OBPs in chemical communication is less clear than that described for MUPs. In species where OBPs are produced in the liver and passaged out by the kidneys via urine, OBPs might have taken the communication roles described for MUPs that are absent from many species. These would, for example, include bank voles [46], and underground-dwelling mole rats [47]. Furthermore, humans have lost the genes for MUPs as well as the genes for true X-linked OBPs.

Because OBPs are abundant in nasal and vomeronasal mucosa, Pevsner at al. [48] suggested that OBPs facilitate a transfer of ligands to the olfactory receptors from ligand–OBP complexes and, consequently, they may act as deactivators by removing the ligands from chemosensory receptors and also scavenge for cytotoxic and genotoxic compounds. Taylor et al. [49] suggested that OBPs may yield a prolongation of a signal activity by a prolonged retention and consecutive release of ligands that are encapsulated by these proteins.

On the basis of the calculation of hydrophobicity index depicted in Figure 1, the biochemical properties of MUPs and OBPs seem rather complementary. Owing to more hydrophobic residues, MUPs are more specialized to bind non-polar volatiles in their pocket than OBPs. However, they have also non-overlapping pI, suggesting that MUPs and OBPs may be active in different pH contexts. A major source of periodic acidification of nasal mucosa is dissolution of carbon dioxide which regularly changes the pH of nasal mucosa during ventilation. Moreover, less hydrophobic OBPs may be important in filtering non-specific or water-degraded ligands to increase the efficiency of relevant signal processing by chemosensory receptors.

Conclusions

Mouse lipocalins are multipurpose protein transporters of various types of ligands, thus manifesting their adaptive roles from the early evolutionary stages of living organisms. Mouse lipocalins involve at least 55 coding genes with most of them organized in duplicated gene clusters. Individual lipocalin members are specialized for particular functions (e.g. immunity, excretion of chemical signals), whereas for others a myriad of potential overlapping functions have been suggested or found experimentally (e.g. excretion of chemical signals along with their roles in activation and deactivation of receptors, and/or scavenging toxic substances [50]).

Funding

This research was supported by the Grant Agency of the Czech Republic (GACR) [grant number P506/12/1046], and by the project BIOCEV – Biotechnology and Biomedicine Centre of the Academy of Sciences and Charles University in Prague [grant number CZ.1.05/1.1.00/02.0109], from the European Regional Development Fund. P.H. is financed by the European Social Fund and the state budget of the Czech Republic [project number CZ.1.07/2.3.00/30.0061].

References

1 Flower, D. (1996) The lipocalin protein family: structure and function. Biochem. J. **318**, 1–14 PubMed
2 Salier, J.-P. (2000) Chromosomal location, exon/intron organization and evolution of lipocalin genes. Biochim. Biophys. Acta **1482**, 25–34 CrossRef PubMed
3 Ganfornina, M.D., Gutierrez, G., Bastiani, M. and Sanchez, D. (2000) A phylogenetic analysis of the lipocalin protein family. Mol. Biol. Evol. **17**, 114–126 CrossRef PubMed
4 Bishop, R.E., Cambillau, C., Privé, G.G., His, D., Tillo, D. and Tillier, E.R.M. (2006) Bacterial lipocalins: origin, structure, and function. In Lipocalins (Akerström, B., Borregaard, N., Flower, D.R. and Salier, J.-P., eds), pp. 28–40, Landes Bioscience, Georgetown
5 Charron, J.B.F. and Sarhan, F. (2006) Plant lipocalins. In Lipocalins (Akerström, B., Borregaard, N., Flower, D.R. and Salier, J.-P., eds), pp. 41–48, Landes Bioscience, Georgetown
6 Ganfornina, M.D., Do Carmo, S., Lora, J.M., Torres-Schumann, S., Vogel, M., Allhorn, M., González, C., Bastiani, M.J., Rassart, E. and Sanchez, D (2008) Apolipoprotein D is involved in the mechanisms regulating protection from oxidative stress. Aging Cell **7**, 506–515 CrossRef PubMed
7 Sharrow, S.D., Vaughn, J.L., Žídek, L., Novotny, M.V. and Stone, M.J. (2002) Pheromone binding by polymorphic mouse major urinary proteins. Protein Sci. **11**, 2247–2256 CrossRef PubMed
8 Novotny, M.V., Ma, W., Wiesler, D. and Zídek, L. (1999) Positive identification of the puberty-accelerating pheromone of the house mouse: the volatile ligands associating with the major urinary protein. Proc. Biol. Soc. **266**, 2017–2022 CrossRef
9 Sam, M., Vora, S., Malic, B., Ma, W., Novotny, M.V. and Buck, L.B. (2001) Odorants may arouse instinctive behaviours. Nature **412**, 142 CrossRef PubMed
10 Novotny, M.V., Ma, W., Zidek, L. and Daev, E. (1999) Recent biochemical insights into puberty acceleration, estrus induction, and puberty delay in the house mouse. In Advances in Chemical Communication in Vertebrates (Johnston, R.E., Müller-Schwarze, D. and Sorensen, P.W., eds), pp. 99–116, Plenum Press, New York CrossRef
11 Roberts, S.A., Simpson, D.M., Armstrong, S.D., Davidson, A.J., Robertson, D.H., McLean, L., Beynon, R.J. and Hurst, J.L. (2010) Darcin: a male pheromone that stimulates female memory and sexual attraction to an individual male's odour. BMC Biol. **8**, 75 CrossRef PubMed
12 Roberts, S.A., Davidson, A.J., McLean, L., Beynon, R.J. and Hurst, J.L. (2012) Pheromonal induction of spatial learning in mice. Science **338**, 1462–1465 CrossRef
13 Jones, D.T., Taylor, W.R. and Thornton, J.M. (1992) The rapid generation of mutation data matrices from protein sequences. Comput. Appl. Biosci. **8**, 275–282 PubMed
14 Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and Kumar, S. (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. **28**, 2731–2739 CrossRef PubMed
15 Logan, D.W., Marton, T.F. and Stowers, L. (2008) Species specificity in major urinary proteins by parallel evolution. PLoS ONE **3**, e3280 CrossRef PubMed
16 Stopková, R., Hladovcová, D., Kokavec, J., Vyoral, D. and Stopka, P. (2009) Multiple roles of secretory lipocalins (MUP, OBP) in mice. Folia Zool. **58**, 29–40
17 Miyawaki, A., Matsushita, F. and Mikoshiba, K. (1994) Possible pheromone-carrier function of two lipocalin proteins in the vomeronasal organ. EMBO J. **13**, 5835–5842 PubMed
18 Fumagalli, M., Sironi, M., Pozzoli, U., Ferrer-Admetlla, A., Pattini, L. and Nielsen, R. (2011) Signatures of environmental genetic adaptation pinpoint pathogens as the main selective pressure through human evolution. PLoS Genet. **7**, e1002355 CrossRef PubMed
19 Whitman, W.B., Coleman, D.C. and Wiebe, W.J. (1998) Prokaryotes: the unseen majority. Proc. Natl. Acad. Sci. U.S.A. **95**, 6578–6583 CrossRef PubMed
20 Qin, J., Li, R., Raes, J., Arumugam, M., Burgdorf, K.S., Manichanh, C., Nielsen, T., Pons, N., Levenez, F., Yamada, T. et al. (2010) A human gut microbial gene catalogue established by metagenomic sequencing. Nature **464**, 59–65 CrossRef PubMed
21 Funkhouser, L.J. and Bordenstein, S.R. (2013) Mom knows best: the universality of maternal microbial transmission. PLoS Biol. **11**, e1001631 CrossRef PubMed
22 Porcheron, G., Garenaux, A., Proulx, J., Sabri, M. and Dozois, C.M. (2013) Iron, copper, zinc, and manganese transport and regulation in pathogenic Enterobacteria: correlations between strains, site of infection and the relative importance of the different metal transport systems for virulence. Front. Cell Infect. Microbiol. **3**, 90 CrossRef PubMed
23 Goetz, D.H., Holmes, M.A., Borregaard, N., Bluhm, M.E., Raymond, K.N. and Strong, R.K. (2002) The neutrophil lipocalin NGAL is a bacteriostatic agent that interferes with siderophore-mediated iron acquisition. Mol. Cell **10**, 1033–1043 CrossRef PubMed
24 Flo, T.H., Smith, K.D., Sato, S., Rodriguez, D.J., Holmes, M.A., Strong, R.K., Akira, S. and Aderem, A. (2004) Lipocalin 2 mediates an innate immune response to bacterial infection by sequestrating iron. Nature **432**, 917–921 CrossRef PubMed
25 Happel, K.I., Zheng, M., Young, E., Quinton, L.J., Lockhart, E., Ramsay, A.J., Shellito, J.E., Schurr, J.R., Bagby, G.J., Nelson, S. and Kolls, J.K. (2003) Cutting edge: roles of Toll-like receptor 4 and IL-23 in IL-17 expression in response to *Klebsiella pneumoniae* infection. J. Immunol. **170**, 4432–4436 CrossRef PubMed
26 Lindahl, M., Stahlbom, B. and Tagesson, C. (1999) Newly identified proteins in human nasal and bronchoalveolar lavage fluids: potential biomedical and clinical applications. Electrophoresis **20**, 3670–3676 CrossRef PubMed
27 Strong, R.K. (2006) Siderocalins. In Lipocalins (Akerström, B., Borregaard, N., Flower, D.R. and Salier, J.-P., eds), pp. 83–98, Landes Bioscience, Georgetown
28 Fluckinger, M., Haas, H., Merschak, P., Glasgow, B.J. and Redl, B. (2004) Human tear lipocalin exhibits antimicrobial activity by scavenging microbial siderophores. Antimicrob. Agents Chemother. **48**, 3367–3372 CrossRef PubMed
29 Lechner, M., Wojnar, P. and Redl, B. (2001) Human tear lipocalin acts as an oxidative-stress-induced scavenger of potentially harmful lipid peroxidation products in a cell culture system. Biochem. J. **356**, 129–135 CrossRef PubMed
30 Fukuhara, A., Yamada, M., Fujimori, K., Miyamoto, Y., Kusumoto, T., Nakajima, H. and Inui, T. (2012) Lipocalin-type prostaglandin D synthase protects against oxidative stress-induced neuronal cell death. Biochem. J. **443**, 75–84 CrossRef PubMed
31 Joo, M., Kwon, M., Sadikot, R.T., Kingsley, P.J., Marnett, L.J., Blackwell, T.S., Peebles, Jr, R.S., Urade, Y. and Christman, J.W. (2007) Induction and function of lipocalin prostaglandin D synthase in host immunity. J. Immunol. **179**, 2565–2575 CrossRef PubMed
32 Ligresti, G., Aplin, A.C., Dunn, B.E., Morishita, A. and Nicosia, R.F. (2012) The acute phase reactant orosomucoid-1 is a bimodal regulator of angiogenesis with time- and context-dependent inhibitory and stimulatory properties. PLoS ONE **7**, e41387 CrossRef PubMed
33 Fournier, T., Medjoubi-N, N. and Porquet, D. (2000) Alpha-1-acid glycoprotein. Biochim. Biophys. Acta **1482**, 575–571 CrossRef
34 Petrak, J., Myslivcová, D., Halada, P., Cmejla, R., Cmejlová, J., Vyoral, D. and Vulpe, C.D. (2007) Iron-independent specific protein expression pattern in the liver of HFE-deficient mice. Int. J. Bioch. Cell Biol. **39**, 1006–1015 CrossRef
35 Shiao, M.S., Chang, A.Y., Liao, B.Y., Ching, Y.H., Lu, M.Y., Chen, S.M. and Li, W.H. (2012) Transcriptomes of mouse olfactory epithelium reveal sexual differences in odorant detection. Genome Biol. Evol. **4**, 703–712 CrossRef PubMed
36 Finlayson, J.S., Asofsky, R., Potter, M. and Runner, C.C. (1965) Major urinary protein complex of normal mice: origin. Science **149**, 981–982 CrossRef PubMed
37 Szoka, P.R. and Paigen, K. (1978) Regulation of mouse major urinary protein production by the *MUP-A* gene. Genetics **90**, 597–612 PubMed
38 Hurst, J.L., Payne, C.E., Nevison, C.M., Marie, A.D., Humphries, R.E., Robertson, D.H., Cavaggioni, A. and Beynon, R.J. (2001) Individual recognition in mice mediated by major urinary proteins. Nature **414**, 631–634 CrossRef PubMed
39 Stopková, R., Stopka, P., Janotová, K. and Jedelsky, P.L. (2007) Species-specific expression of major urinary proteins in the house mice (*Mus musculus musculus* and *Mus musculus domesticus*). J. Chem. Ecol. **33**, 861–869 CrossRef PubMed
40 Stopka, P., Janotová, K. and Heyrovsky, D. (2007) The advertisement role of major urinary proteins in mice. Physiol. Behav. **91**, 667–670 CrossRef PubMed
41 Janotová, K. and Stopka, P. (2011) The level of major urinary proteins is socially regulated in wild *Mus musculus musculus*. J. Chem. Ecol. **37**, 647–656 CrossRef PubMed
42 Janotová, K. and Stopka, P. (2009) Mechanisms of chemical communication: the role of major urinary proteins. Folia Zool. **58**, 41–55

43 Bignetti, E., Cavaggioni, A., Pelosi, P., Persaud, K.C., Sorbi, R.T. and Tirindelli, R. (1985) Purification and characterisation of an odorant-binding protein from cow nasal tissue. Eur. J. Biochem. **149**, 227–231 CrossRef PubMed
44 Pevsner, J., Hou, V., Snowman, A.M. and Snyder, S.H. (1990) Odorant-binding protein. J. Biol. Chem. **265**, 6118–6125 PubMed
45 Briand, L., Huet, J.-C., Perez, V., Lenoir, G., Nespoulous, C., Boucher, Y., Trotier, D. and Pernollet, J.-C. (2000) Odorant and pheromone binding by aphrodisin, a hamster aphrodisiac protein. FEBS Lett. **476**, 179–185 CrossRef PubMed
46 Stopková, R., Zdrahal, Z., Ryba, S., Sedo, O., Sandera, M. and Stopka, P. (2010) Novel OBP genes similar to hamster aphrodisin in the bank vole, *Myodes glareolus*. BMC Genomics **11**, 45 CrossRef PubMed
47 Hagemeyer, P., Begall, S., Janotová, K., Todrank, J., Heth, G., Jedelsky, P.L., Burda, H. and Stopka, P. (2011) Searching for major urinary proteins (MUPs) as chemosignals in urine of subterranean rodents. J. Chem. Ecol. **37**, 687–694 CrossRef PubMed
48 Pevsner, J., Sklar, P.B. and Snyder, S.H. (1986) Odorant-binding protein: localization to nasal glands and secretions. Proc. Natl. Acad. Sci. U.S.A. **83**, 4942–4946 CrossRef PubMed
49 Taylor, A.J., Cook, D.J. and Scott, D.J. (2008) Role of odorant binding proteins: comparing hypothetical mechanisms with experimental data. Chem. Percept. **1**, 153–162 CrossRef
50 Grolli, S., Merli, E., Conti, V., Scaltriti, E. and Ramoni, R. (2006) Odorant binding protein has the biochemical properties of a scavenger for 4-hydroxy-2-nonenal in mammalian nasal mucosa. FEBS J. **273**, 5131–5514 CrossRef PubMed

Received 4 March 2014
doi:10.1042/BST20140053

frontiers in Ecology and Evolution

ORIGINAL RESEARCH
published: 28 April 2016
doi: 10.3389/fevo.2016.00047

Mouse Lipocalins (MUP, OBP, LCN) Are Co-expressed in Tissues Involved in Chemical Communication

*Romana Stopková[1], David Vinkler[1], Barbora Kuntová[1], Ondrej Šedo[2,3], Tomáš Albrecht[1], Jan Suchan[1], Kateřina Dvořáková-Hortová[1], Zbyněk Zdráhal[2,3] and Pavel Stopka[1]**

[1] BIOCEV Group, Department of Zoology, Faculty of Science, Charles University in Prague, Prague, Czech Republic, [2] Research Group Proteomics, Faculty of Science, Central European Institute of Technology, Masaryk University, Brno, Czech Republic, [3] Faculty of Science, National Centre for Biomolecular Research, Masaryk University, Brno, Czech Republic

Chemical communication is mediated by signal production and signal perception and in house mice (*Mus musculus*), both processes involve lipocalin proteins (OBP, MUP, LCN) that transport volatiles and protect them in tissues where they are produced. However, potential roles of lacrimal, nasal, and salivary lipocalins are still not well known. We aimed to determine the expression of the recently described family of odorant binding proteins (*Obp*), along with major urinary proteins (*Mup*) across different tissues in wild mice (*Mus musculus*) to assess the importance of these proteins based on their quantity in particular expression sites. We performed qPCR analysis of selected *Mup*, *Lcn, Obp* genes, and predicted *Obp* members to study their expression in selected tissues. We identified new members of the mouse odorant binding protein gene family in two subspecies, *M. m. musculus* and *M. m. domesticus*. We show that *Mup4* and *Mup5* from the phylogenetically older group-A are co-expressed with *Obp*s in orofacial tissues. We also identified a sexually dimorphic pattern of female-biased *Obp7* and male-biased *Mup4* expression in lacrimal glands. OBPs, MUPs, and LCNs are produced in parallel, which may function to widen the spectrum of bound ligands, potentially including the degradation products of olfactory signals and/or toxic compounds. Moreover, our study demonstrates that several pheromone transporters from the lipocalin family are co-expressed in the nasal and lacrimal tissues of mice with the newly detected OBPs that further expand the already diverse mouse lipocalin family.

Keywords: lipocalin, odorant, chemical communication, *Mus musculus*, olfaction

OPEN ACCESS

Edited by:
Carla Mucignat, University of Padova, Italy

Reviewed by:
Patricia Nagnan-Le Meillour, Institut National de la Recherche Agronomique, France
Dustin J. Penn, Veterinary University Vienna, Austria
Roberto Ramoni, University of Parma, Italy

****Correspondence:***
Pavel Stopka
pstopka@natur.cuni.cz

Specialty section:
This article was submitted to Chemical Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: *26 February 2016*
Accepted: *15 April 2016*
Published: *28 April 2016*

Citation:
Stopková R, Vinkler D, Kuntová B, Šedo O, Albrecht T, Suchan J, Dvořáková-Hortová K, Zdráhal Z and Stopka P (2016) Mouse Lipocalins (MUP, OBP, LCN) Are Co-expressed in Tissues Involved in Chemical Communication. Front. Ecol. Evol. 4:47. doi: 10.3389/fevo.2016.00047

BACKGROUND

John Maynard Smith and David Harper defined signal as "...any act or structure which alters the behaviour of other organisms, which evolved because of that effect, and which is effective because the receiver's response has also evolved" (Maynard Smith and Harper, 2003). The house mouse (*Mus musculus*) uses a system of volatile pheromones (Mucignat-Caretta et al., 2010) and their transporters from the lipocalin protein family that together form a signal (Novotny et al., 1985). Because volatiles degrade in water solutions (Kwak et al., 2013), their life span largely depends on lipocalins that protect them (Hurst et al., 1998; Timm et al., 2001), and transport them in secretory fluids (Flower, 1996) to an outside world. The signals have strong effects on the reproductive success

of the signaler (Thonhauser et al., 2013) due to strong effects on reproductive physiology of the receiver (Whitten et al., 1968; Roberts et al., 2004; Stopka et al., 2007; Janotova and Stopka, 2011) through chemosensory receptors of the main olfactory and vomeronasal organs (Moss et al., 1997; Luo and Katz, 2004).

Since the discovery of the structure and function of olfactory receptors GPCRs—G-protein coupled receptors (Buck and Axel, 1991), research on chemical communication has concentrated on signal reception by nasal and vomeronasal chemosensory neuronal receptors, and on lipocalin transporters of pheromones. Lipocalins generally function to sequester hydrophobic volatiles and transport them in their eight-stranded beta barrel structure (Timm et al., 2001; Sharrow et al., 2002). Volatiles specifically bind to receptors of chemosensory neurons when released (Tirindelli et al., 1998; Novotny, 2003). In mice, the functions of lipocalin transporters are not well understood and most studies focused on the major urinary proteins (MUPs), which are expressed in the liver and transport volatile odor/organic compounds (VOCs) to the urine (Shahan and Derman, 1984; Shahan et al., 1987a,b; Stopková et al., 2007). MUPs have also been reported to be expressed in several tissues other than the liver (Shaw et al., 1983; Shahan et al., 1987a; Cavaggioni et al., 1999; Utsumi et al., 1999; Karn and Laukaitis, 2011), though their functions are not understood.

Mup genes have recently duplicated in rodents, and in house mice they form a cluster of 21 coding genes (and a similar number of pseudogenes), which can be divided into two groups, the group-A (ancestral), containing *Mup3, Mup4, Mup5*, Mup6, *Mup20* (or "Darcin"), and *Mup21* and the group-B, consisting of 15 other *Mup*s sharing almost 99% sequence identity: *Mup1, Mup2, Mup7-Mup19* (Logan et al., 2008; Mudge et al., 2008), reviewed in Janotová and Stopka (2009), Stopková et al. (2009), and Phelan et al. (2014). The level of urinary MUP production is socially regulated in C57BL/6 laboratory mice (Stopka et al., 2007) and wild living *M. m. musculus* (Janotova and Stopka, 2011) and *M. m. domesticus* (Cunningham et al., 2013) mice. Furthermore, male *M. m. musculus* up-regulated urinary MUP production when caged with a female, but down-regulated when caged with a male. Down-regulation of MUPs was more pronounced in males that were defeated in a male–male encounter (Janotova and Stopka, 2011). Furthermore, social experience of parents can regulate MUP expression level in subsequent generations through epigenetic effects (Nelson et al., 2013).

Specific roles limited to a single urinary MUP were attributed only to a major urinary protein MUP20 (or "Darcin"; a group-A MUP) expressed in males, which attracts females and aids spatial learning (Roberts et al., 2010, 2012). Remaining MUPs were supposed to present an individual "barcode" signal due to differences in urinary MUP profiles (Hurst et al., 2001). However, a recent study with sufficient sample sizes shows that MUP profiles of wild male house mice (*M. m. musculus*) are not individually unique. They are not highly stable but instead are dynamic over time with significant changes after puberty and during adulthood (Thoß et al., 2015), thus challenging the "barcode" hypothesis. Moreover, the variation in pheromone affinities of the urinary MUP isoforms provides low support for the proposal that heterogeneity in MUPs plays a role in regulating profiles of available pheromones (Sharrow et al., 2002).

Another group of lipocalins that is thought to be involved in chemical communication, but less understood, is a cluster of the odorant binding protein genes (*Obp*). *Obp* genes have also undergone a series of duplications in mice, and they occur in a cluster of six genes and two pseudogenes on the X chromosome (Stopková et al., 2009, 2014, see **Figure 1A**). Whilst the *Mup* genes are abundant only in house mice and rats (*Rattus norvegicus*) and rarely found in other species of mammals in multiple copies, *Obp* genes occur as a cluster in various mammalian taxa, e.g., porcupines (*Hystrix cristata*) (Felicioli et al., 1993), bank voles (*Myodes glareolus*) (Stopkova et al., 2010), elephants (Lazar et al., 2002), cows (*Bos taurus*) (Bignetti et al., 1985), boar (*Sus scrofa*) (Spinelli et al., 1998; Nagnan-Le Meillour et al., 2014), and potentially also mole rats (*Fukomys anselli*, *F. kafuensis*) (Hagemeyer et al., 2011). One OBP member (i.e., Aphrodisin) has been shown to be major pheromone transporter in vaginal flushes of hamsters (*Cricetus cricetus*). Interestingly, pigs have OBPs and SAL. SAL is the major salivary protein in pigs with affinity to steroids and to 2-isobutyl-3-methoxypyrazine, it is phylogenetically close to MUPs and is expressed by the male submaxillary glands (Marchese et al., 1998). Moreover, three of the six predicted OBP members described (Stopková et al., 2009, 2014) were also corroborated with MS techniques in the tear and saliva proteomes of the laboratory mouse C57BL/6 (Karn and Laukaitis, 2015), though the authors did not further specify detected OBP variants. Therefore, one of the aims of our paper was to detect potential expression sites of mRNAs coding OBPs that were found in the mouse saliva.

Because mice typically begin social interactions by investigating facial and mouth areas (Luo et al., 2003), we may assume that tear and salivary lipocalins secrete chemical signals, whilst nasal and vomeronasal lipocalins activate and/or deactivate chemosensory GPCR receptors. In addition to their function in chemical communication, some lipocalins also have important roles in innate immune responses (Fluckinger et al., 2004; Stopková et al., 2014). We have previously suggested that chemical communication and immunity have been shaped by similar evolutionary forces because the nasal cavity is a place of pathogen recognition via lymphoid tissues and signal perception via chemosensory neurons (Stopková et al., 2009, 2014). Moreover, lipocalins may have as yet another function. The "toxic waste hypothesis" states that various lipocalins are involved in removing toxic waste from the body (Stopková et al., 2009; Kwak et al., 2011) and that some of the compounds might have been constituting a signal under selection (Stopková et al., 2009). The toxic waste disposal role has been experimentally demonstrated in a recent paper (Kwak et al., 2016) where mice loaded with an industrial chemical, 2,4-di-tert-butylphenol (DTBP) used MUPs for a consequent detoxification (Kwak et al., 2016). To conclude, lipocalins are ubiquitous proteins with diverse functions and multiple sites of their expression.

In this study, we investigated potential differences in the expression of selected lipocalins in two sub-species of the house

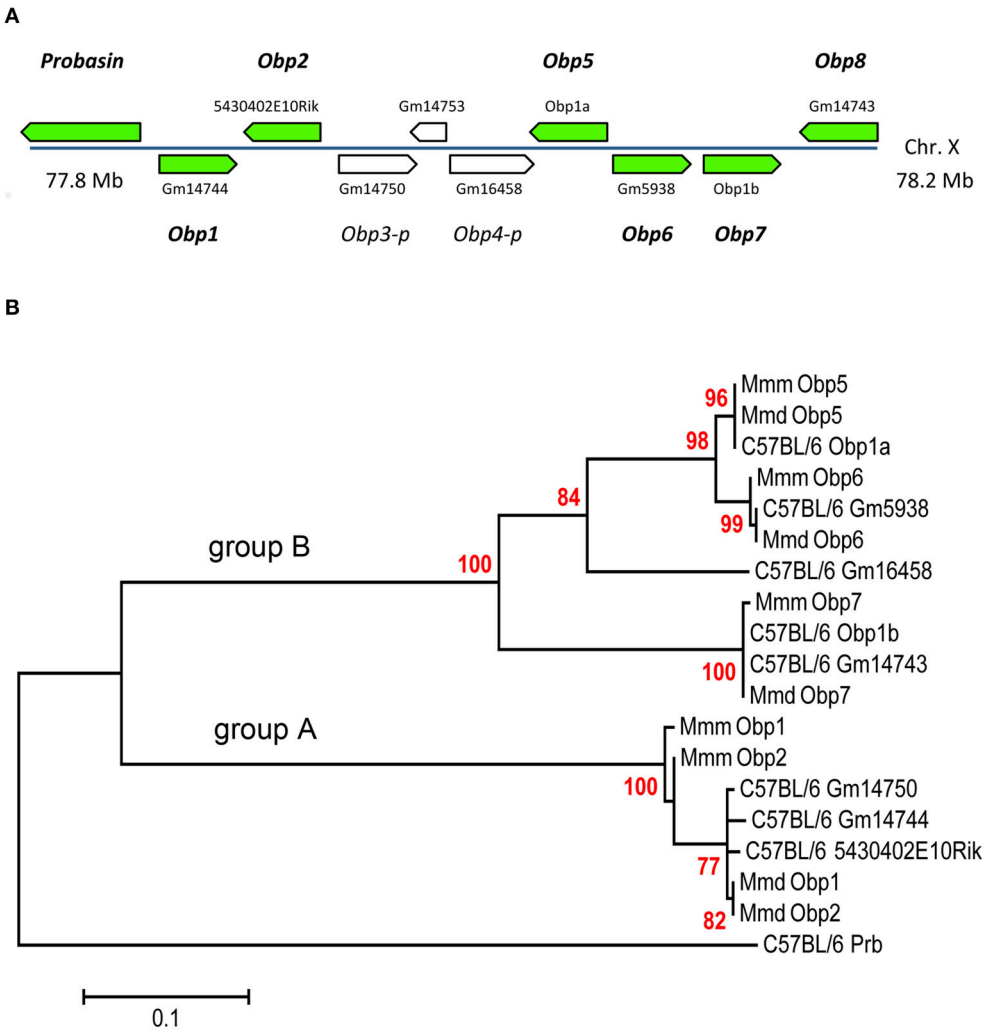


FIGURE 1 | The mouse *Obp* cluster (A), and Phylogenetic analysis of novel OBPs by Maximum Likelihood method (B). Green arrows **(A)** indicate position of coding genes mapped on C57BL/6 X-chromosome with specific codes from the mouse genome. Consecutive numbering in italics (*Obp1* to *Obp8*) represents alternative names for corroborated transcripts under this study in wild mice. This nomenclature also involves two pseudogenes (*Obp3-p, Obp4-p*) with positions indicated by white arrows. Pseudogene Gm14753 is not involved in numbering because it is a processed retroelement similar to actin and not *Obp*.

mouse, *Mus musculus musculus* and *M. m. domesticus*. This could be a starting point to determine how these proteins evolve through speciation (Hiadlovska et al., 2013), and their potential influence on sub-species recognition (Smadja and Ganem, 2002, 2008) and aggression (Dureje et al., 2011). These two sub-species have been previously found to vary quantitatively in the abundance of male VOCs (Mucignat-Caretta et al., 2010) and MUP expression between sexes (Stopková et al., 2007) with differences in the beta-barrel residues under selection (Karn and Laukaitis, 2012). Thus, we have identified new members of the odorant binding protein family and focused on the level and locations of expression of soluble lipocalins. It is our hope that investigating lipocalins in different tissues, and not only in the urine, will provide a better understanding of this fascinating and complex family of carrier proteins.

METHODS

Ethical Standards

All animal procedures were carried out in strict accordance with the law of the Czech Republic paragraph 17 no. 246/1992 and the local ethics committee of the Faculty of Science of Charles University in Prague specifically approved this study in accordance with accreditation no. 27335/2013-17214 valid through 2019. Animals were sacrificed by cervical dislocation.

Samples

The total of 12 individuals (i.e., six males and six females) was studied in this experiment with six individual *M m. domesticus* from Hattingen (51°20′39.84″N, 7°12′06.38″E) and Ruther (51°23′01.0″N, 6°57′48.9″E) and six individual *Mus m. musculus* from Jičín (50°28′18.802″N, 15°22′31.667″E). Individual mice

were kept for 6 weeks following capture in the accredited mouse facility with food and water provided *ad libitum* and on a 12:12-h light cycle with lights off at 1900 h. Samples for 2DE were collected from the oral cavity with 100 μl of 0.9% saline water repeatedly flushing in and out with a pipette. Samples were immediately acetone precipitated and used in further steps.

When salivary samples were collected, all specimens were sacrificed by cervical dislocation and tissue samples were collected from each animal. Tissue samples were obtained from preputial/clitorial glands, liver, lacrimal and Harderian glands, submandibular (salivary) glands, olfactory epithelia, vomeronasal organ, and Nasal-associated lymphoid tissue (NALT). NALT is the paired lymphoid organ (Kiyono and Fukuyama, 2004), and it was isolated from the upper mouse jaw by peeling away the palate where NALT was localized bilaterally on the posterior side.

2DE-Page Analysis

Two dimensional polyacrylamide electrophoresis (2DE) was performed with IEF cell (Bio-rad®) and Protean II electrophoresis system. For the first dimension 12 μg of proteins was applied to Bio-Rad 11 cm strips (pI: 3.9–5.1). Isoelectric focusing was performed after passive rehydration at room temperature and run at 50 V for 9 h, 250 V (rapid) for 15 min, 8000 V (rapid) for 1 h, 8000/30,000 V/h, and finished at 500 V until further step. For the second- dimension separation—strips were equilibrated for 10 min in 45 mM Tris base (pH 7.0) containing 6 M urea, 1.6 SDS, 30% glycerol, and 130 mM dithiothreitol, and then re-equilibrated for 10 min in the same buffer containing 135 mM iodoacetamide in place of dithiothreitol. The strips were then placed on Criterion (dodeca) precast 12–20% gels along with unstained molecular standards in a separate well. Second dimension gels were run at constant current—50 mA for 1 h, 100 mA for 1 h and 150 mA for 1.5 h at 10°C. After electrophoresis, the gels were stained with the Colloidal Coomassie G-250 stain (Bio-rad). All spots in the range 15–30 kDa were excised with a Bio-rad Spot Cutter.

MALDI-MS/MS Analysis

The most abundant protein spots were selected for the analysis and excised from 2-DE gels from 12 individuals. Gel pieces were destained by alternative washing steps using 50 mM ammonium bicarbonate and acetonitrile (i.e., provided in detail in **Data Sheet 1**). After destaining, the proteins in gel pieces were incubated with trypsin (sequencing grade, Promega) at 37°C for 2 h. Digested peptides were extracted from gels using 50% ACN solution with 5% formic acid. MALDI- MS/MS analyses were performed on an Ultraflex III mass spectrometer (Bruker Daltonik, Bremen, Germany). Peptide maps were acquired inreflectron positive mode (25 kV acceleration voltage) with 800 laser shots. Peaks within 700–4000 Da mass range and minimum S/N 10 were picked out for MS/MS analysis employing LID-LIFT arrangement with 600 laser shots for each peptide.

CHCA was used as the matrix in combination with AnchorChip target to enhance measurement sensitivity. Sample (1 μl) was mixed with matrix solution on the target in a 2:1 ratio. Known autoproteolytic products of trypsin were used for internal calibration of digested peptides. In the absence of these products, an external calibration procedure was employed, using a mixture of seven peptide standards (Bruker Daltonik) covering the mass range of 1000–3100 Da. The Flex Analysis 3.0 and MS Biotools 3.1 (Bruker Daltonik) software were used for data processing.

Data Processing

MASCOT 2.2 (MatrixScience, London, UK) search engine was used for processing the MS/MS data under standard settings with significance threshold $p < 0.05$. Database searches were done against the NCBI protein database (Release 20101113) without taxonomic restriction. Mass tolerances of peptide precursors and MS/MS fragments were set to 60 ppm and 0.7 Da, respectively. Trypsin specificity with possibility of semitryptic cleavage, oxidation (M), carbamidomethylation (C) and pyro-Glu (Q, N-term) as optional modifications and up to two enzyme miscleavages were set for all searches. Protein identifications based on one or more unique peptides with significant score (under the settings—59 or higher) were accepted. See more details in **Data Sheet 1**.

Real-Time PCR Analysis

Immediately after resection, each tissue sample was placed into Eppendorff tube with a mixture of 1 ml of Trizol (TRIzol Reagent–Invitrogen) and glass pellets, and homogenized using a homogenizer (MM200–Retsch). RNA was isolated using standard Trizol protocol and followed by cDNA synthesis using First strand cDNA synthesis kit (Fermentas). RNA was assessed from the ratio of the optical densities at 260 and 280 nm, and the RNA integrity was assessed with 1% agarose gel containing ethidium bromide. One microgram of total RNA (DNase treated) was used for the synthesis of single-stranded cDNA according to a first-strand cDNA synthesis protocol (Fermentas UAB, Vilnius, Lithuania) with RevertAid™M-MulV Reverse Transcriptase and oligo(dT)18 primer.

Real-time PCR was performed on a Light Cycler 480 (Roche Applied Sciences) using specific dual hydrolysation probe method (Universal Probe–Roche Applied Sciences) with the Probe Master kit (Roche) and protocol according to the manufacturer's instructions. Specific primers and their respective probes were designed by Universal probe library software (Roche) using our newly provided (i.e., *Obp*) and NCBI reference sequences. Intron-spanning assay and multiplex PCR condition with reference gene (Gapdh) were selected. The resulting primers and probes are provided in **Table 1**. Moreover, most group-B *Mup*s are almost 99% similar and it is difficult on the level of transcript to find a probe that would differentiate between different group-B *Mup*s. Thus, most urinary *Mup*s including *Mup2* is included in **Figure 3** within the category MUP-B (detected with universal group-B *Mup* primers).

PCR amplification was performed with the following conditions: initial denaturation at 95°C for 10 min, followed by 40 cycles consisting of denaturation at 95°C for 10 s, annealing at 60°C for 10 s where fluorescence was acquired, and elongation at 72°C for 5 s. Each sample was measured in triplicate. The data used for calculation are the means of Cq (i.e., cycle of quantitation

TABLE 1 | Primers and probes used in Real-time qPCR analysis.

Gene	Forward primer	Reverse primer	# UPL probe
Atp5b	ggcacaatgcaggaaagg	tcagcaggcacatagatagcc	77
Lcn11	agaacattgtggacctttctt	ggagaagggtgggtcagc	29
Lcn4	aatgtaggaattcgtttgcag	gagagtatggccccaaaagg	82
Mup-B	gacctatccaatgccaatcg	tggataggaagggatgatgg	47
Mup21	gggaaggaactttaatgtaga	ccacaaaagctctcatgctg	110
Mup4	atggcctgagcctccagt	gctgtatcgatcggaagagag	67
Mup5	gaatgaagaatggcctgagc	caccccatgctgtatggaa	67
Obp1	gcgcacccttttacatagctg	acgctctcaggtctccattc	39
Obp5	ggaccatggaaaactgttgc	cagttctccacctctctctatcttg	146
Obp6	cctgtcctgagtaatgatcttct	ctgattccacaagtcatgaggtt	18
Obp7	tcaagcaaaatggacaatgc	tgccatcttcttgcttataccc	114

in Roche software) values of triplicate samples. The variation in triplicate values never exceeded 0.5 Cq in our samples.

The level of mRNA of the target gene (*Obp* etc.,) in each sample was calculated relative to the reference gene (*Gapdh*) amplified in the same well. A calibration curve was generated for each pair of primers using 10-fold serial dilution of cDNA to assess the value for PCR efficiency (E). In all cases E was not lower than 0.9 (i.e., 90% efficiency of PCR reaction). *E*-values were than used in the formula (The Efficiency sensitive model Pfaffl, 2001) used for the calculation of relative expression (RE), i.e., normalized mRNA abundance:

$$\mathrm{RE} = (1 + \mathrm{E}_{\mathrm{reference}})^{\mathrm{Cp}_{\mathrm{reference}}} / (1 + \mathrm{E}_{\mathrm{target}})^{\mathrm{Cp}_{\mathrm{target}}}$$

Non-template and non-RT reactions were used as controls. For the analysis of expression patterns via hierarchical clustering we used R software. Our hierarchical clustering utilized Euclidian distance metric on log2 transformed data and complete linkage method. The mixed-model approaches, ANOVA, *t*-test, Shapiro-Wilk's normality test, and Fligner-Killeen test for testing the homogeneity of variances, were also computed and plotted in R (Venables and Smith, 2009). Data for pI values were cross-checked from multiple online resources (Ensembl Genome Browser—www.ensembl.org/, NCBI) and with our recently obtained sequences. Isoelectric point was calculated with ExPASy (http://web.expasy.org/compute_pi/), whilst the index of hydropathy (i.e., GRAVY index) was calculated with Gravy calculator (www.gravy-calculator.de/). Sequence data are provided as additional **Data Sheet 2**, data for calculation of pI and H are provided in **Data Sheet 3**.

Sanger Sequencing

Various primer sets were derived from predicted sequences of the genome mouse C57BL/6 (Stopková et al., 2009, 2014) and used to amplify *Obp* transcripts. Mixed samples from studied orofacial tissues were used for transcript identification. Finally we set up a pair of primers per transcript giving one clear band in the expected area. These sequencing primers covered the whole region from start to stop codon: *Obp1*- (F—CTC TGAACTCCTTCGGAAGGA, R—AAAAGAATCAGTACC ATGGTAGGA), *Obp5*—(F—CTGTAGAAAAGAAAGTCT TGTACCA, R—CATTCAAAAAAGGAAGATCATGAGA), *Obp6*—(F—AAGTCTTGTGCCATAATGGCAA, R—TCAAAA AAAGGAATAACAGGTCGTA), *Obp7*—(F - TGAACATCT CCAGAGGAGCAA, R—GGAAGAAGAGTTTATAGATTA GGCAA). The products were double sequenced (downstream, upstream) with 3130 Genetic Analyzer, Applied Biosystems using either forward or reverse *Obp* primers and with 5 to 10 technical replicates per transcript. Sequences were analyzed using the Sequence Scanner (Applied Biosystem) software and compared to predicted and known sequences from public database NCBI using BLAST. Novel sequences were deposited in GeneBank with accession numbers provided in the results section.

Phylogenetic Analysis of *Obp*s by Maximum Likelihood Method

The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model (Jones et al., 1992). The tree with the highest log likelihood (−1578.6730) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 19 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 284 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011).

RESULTS

Saliva Contains Lipocalins

To detect the expression of lipocalins on the level of proteins, we used the MALDI- MS/MS analyses for protein identification in mouse saliva. In both subspecies, we have identified several abundant lipocalins (**Figure 2**): OBP5 (Odorant binding protein 1a, gi|1835143), LCN11 (Lipocalin 11, gi|154689678), MUP5 (Major urinary protein 5 precursor, gi|113930708), and highly similar group-B MUPs with the most likely identification provided in **Data Sheet 1**. We have also identified a fragment of a putative pheromone transporter VM (Vomeromodulin precursor, gi|70909314) which is a 70 kDa glycoprotein expressed in the posterior septal and vomeronasal glands but not in the mucus of the main olfactory neuroepithelium (Khew-Goodall et al., 1991). The presence of VM (and potentially also OBP5) in the mouse saliva suggests that nasal and oral cavities are functionally connected because proteins expressed

by nasal/vomeronasal tissues are also found in the oral cavity. We have also identified several proteins below 17 kDa size including PIP [Prolactin Inducible Protein—also highly abundant in saliva of the laboratory mouse (Blanchard et al., 2015)], and several unspecified members of ABP (androgen binding protein) family which were recently described in detail in the tear and saliva proteomes of the genome mouse C57BL/6 (Blanchard et al., 2015; Karn and Laukaitis, 2015).

mRNA Sequencing Corroborated Predicted *Obp*s

In contrast to MUPs, the family of OBPs is rather enigmatic with respect to the expression of their predicted members. In studying the poorly-described OBP1a protein found in mouse saliva, we discovered that it is related to a gene cluster that had been incompletely described (Stopková et al., 2014). Therefore, we have sequenced all *Obp* predicted transcripts in wild mice from pooled oro-facial tissues using primers generated from C57BL/6 genomic data and provided specific product names based on their chromosomal position (for MUPs see Logan et al., 2008). All *Obp* transcripts were mapped on the X chromosome of the laboratory mouse C57BL/6 and have been given consecutive names *Obp1-Obp8* (**Figure 1A**). We have also included two pseudogenes (i.e., *Obp3-p, Obp4-p*; classified by Ensembl as unprocessed pseudogenes without a protein product) in our consecutive numbering of *Obp*s as in other rodent taxa these genes may be intact with ORF (i.e., not truncated) and expressed. Thus, we have provided unique *Obp* sequences for feral *M. m. domesticus* (*Obp1*—KJ605385, *Obp2*—KJ605386, *Obp5*—KJ605387, *Obp6*—KJ605388, and *Obp7*—KJ605389), and *M. m. musculus* (*Obp1*—KJ605390, *Obp2*—KJ605391, *Obp5*—KJ605392, *Obp6*—KJ605393, and *Obp7*—KJ605394) and submitted them to GenBank (NCBI).

Phylogenetic Analysis of Novel *Obp* Sequences

All novel OBPs have a feature typical for the entire *Obp* cluster - a specific disulfide bond (Cys38–Cys42), which represents a strong OBP-diagnostic motif CXXXC (Cys-Xaa-Xaa-Xaa-Cys; reviewed in Stopková et al., 2009, 2014). We used our mRNA (i.e., CDS) sequences along with those from C57BL/6 mice to generate the Maximum Likelihood (MLM) tree (**Figure 1B**). The MLM algorithm with 2000 permutations identified *Prb* (Probasin) as the root (i.e., the outgroup to all OBPs). Thus, remaining *Obp*s form two sub-clusters that we decided to name as the group-A and the group-B *Obp*s. Ancestral group-A *Obp*s include *Obp*1 and *Obp*2 (bootstrap = 100). The later evolved group-B *Obp*s include *Obp5*, *Obp6*, *Obp7*, and *Obp8* (bootstrap = 99). The group-B *Obp* sequences perfectly match those predicted transcripts that we extracted from the laboratory mouse genome (**Figure 1A**). However, newly described *Obp1* and *Obp2* from *M. m. musculus* cluster together (bootstrap = 90) and seem to be divergent from *M. m. domesticus* and C57BL/6 (see **Data Sheet 2** for Multiple sequence alignment).

The strong CXXXC motif present in all OBP proteins (including Probasin) is represented by **C**NDD**C** in OBP1 and OBP2, **C**DEG**C** in OBP7 and OBP8, **C**EKE**C** in OBP5 and OBP6. *Obp3-p* pseudogene (if expressed) would belong to the group-A cluster whilst *Obp4-p* would belong to the group-B, **Figure 1B**. Novel *Obp* (transcript) sequences along with the *Mup* sequences downloaded from NCBI were translated and further used for the calculation of hydropathy and pI properties. We also measured the expression of these newly identified genes in numerous tissues.

Hierarchical Clustering Revealed Differential mRNA Expression Across Tissues

We assessed expression of lipocalins in eight tissues using qRT-PCR. We were primarily interested in the location of expression of the newly described OBPs. So we designed primers for all OBPs and for other lipocalins from the list of identified proteins and the VNO-specific LCN4. In the next step, we used hierarchical clustering in R software as a graphical method to show relationships among expression levels of different genes across tissues. Primarily, we focused on the detection of similarities among expression levels in different tissues and averaged across individuals of the two subspecies to cluster particular tissues on the basis of their similar pattern (**Figures 3A,B**).

Hierarchical clustering separated selected tissues according to their pattern of expression into two groups depicted on (upper) X axis in **Figure 3**. Interestingly, in *M. m. domesticus* - olfactory epithelia (OE), lacrimal gland (LG), and nasal-associated lymphoid tissue (NLT) have clustered together in both subspecies (see **Figures 3A,B**), whilst vomeronasal organ (VNO) and other secretory tissues including liver, Harderian gland, submandibular gland, and preputial gland were located on the other branch in *M. m. domesticus* (**Figure 3A**). In *M. m. musculus* VNO clustered together with OE, LG, and NLT (**Figure 3B**), thus suggesting higher VNO activity in this subspecies. This difference is shown in **Figure 3C** where the *M. m. domesticus* matrix is subtracted from that of *M. m. musculus*. The average matrix in **Figure 3D** is a representative matrix with individuals averaged over the two subspecies.

Sexually Dimorphic *Mup*s in the Liver, and Lacrimal *Mup4* and *Obp7*

Mean value of log2 expression levels from the whole data set and standard deviation was 0.759 ± 1.154. Therefore, we opted for a two-fold ($\sim$2 sd) filtering procedure to obtain data with elevated sexual dimorphisms. To our surprise and probably due to the limited sample size, we have detected only three sexually dimorphic genes: the male-biased group-B *Mup*s (i.e., a group of highly similar genes amplified with the primers provided in **Table 1**) in the liver, and unique male-biased lacrimal group-A *Mup4,* and female-biased lacrimal *Obp7*. Only these three groups were further tested.

To control for pseudoreplication of the expression data (i.e., three measurements taken from each individual with the total of 36 measurements) we used a mixed-effect model approach

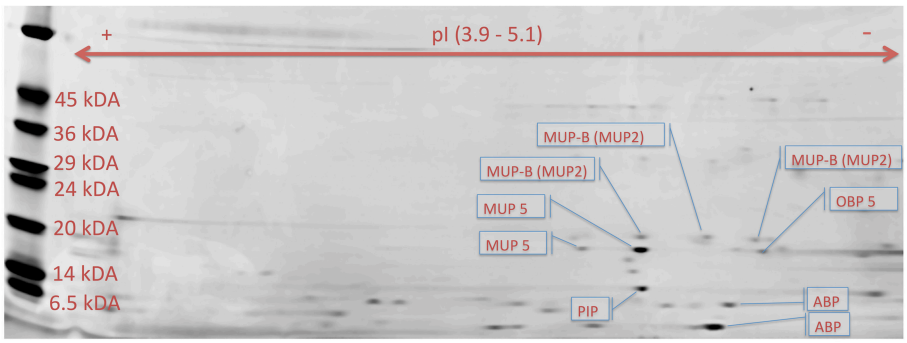


FIGURE 2 | Proteomic search for acidic proteins with hydrophobic beta barrel. Proteomic 2DE analysis on narrow range strips (pI 3.9–5.1) revealed that mouse saliva from a male (*M. m. domesticus*) contains proteins depicted in the figure including OBP5 (MMD11 spot in A1 file), group-A MUP5 (spots: MMD15, MMD17), group-B MUP2 (spots: MMD12-14), unspecified ABPs (spots: MMD18) and prolactin-inducible protein, PIP (spot MMD16). In most gels (i.e., from males and females) we also identified VM (vomeromodulin), LCN11 (lipocalin11). See additional file A1 for a MS report (i.e., section *Mus musculus domesticus*—male).

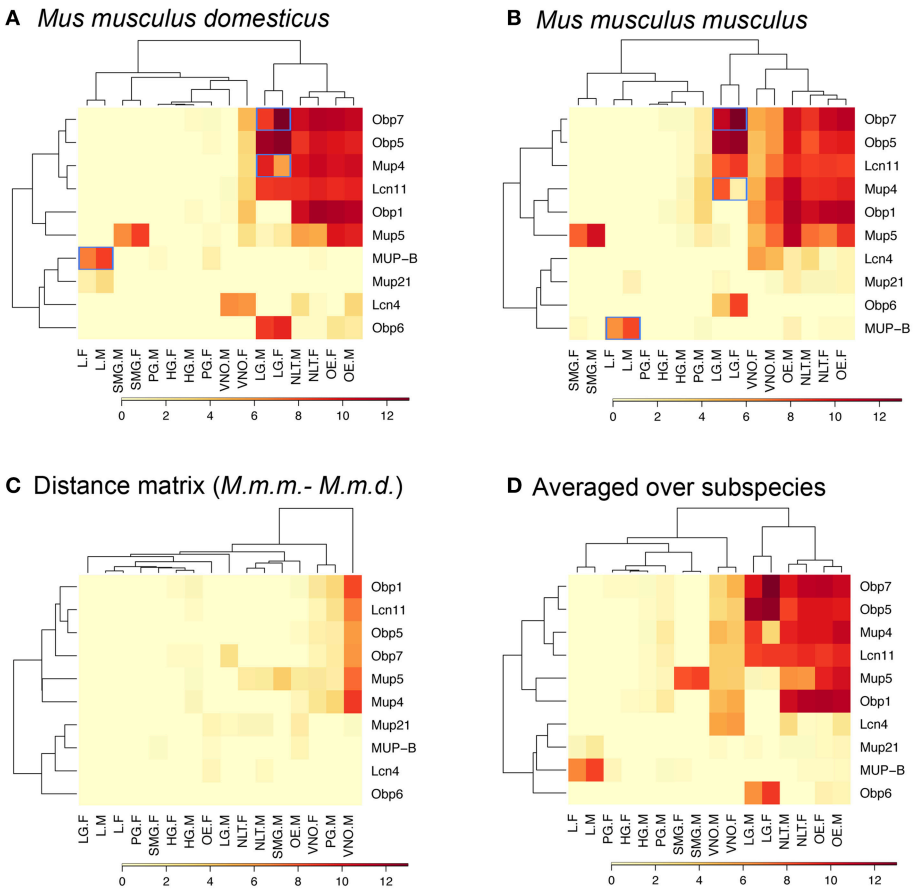


FIGURE 3 | Graphical representation of the qPCR expression pattern. Group-A *Mups* are co-expressed with *Obps* and *Lcns* in sensory tissues and exo-orbital lacrimal glands, whilst the later evolved group-B *Mups* are an outgroup for their specific expression and excretory functions by the liver. Blue-framed are the significantly sexually dimorphic genes (i.e., male-biased group-B *Mups* in the liver, male-biased group-A *Mup4* in lacrimal glands, and female-biased *Obp7* in lacrimal glands). Abbreviations: L, Liver; SMG, submandibular gland; PG, preputial gland; HG, Harderian gland; LG, lacrimal gland; VNO, vomeronasal organ; NLT, NALT / nasal-associated lymphoid tissue; OE, olfactory epithelia; F, females; M, males; Highly expressed genes (~12 fold) are dark red whilst low expression genes are in pale yellow. Heat-maps are provided for each sub-species, **(A)** *M. m. domesticus*, **(B)** *M. m. musculus*, for the distance between them **(C)** and their average **(D)**.

(*nlme* package) assuming normal distribution of the dependent variable, with individuals as random grouping variable (i.e., 12 clusters), and sex, gene, and species as fixed effects. Based on the minimum adequate model (Crawley, 2007) the level of detected sexual dimorphism was highly significant $\Delta df = 9$, L-Ratio = 102.1, $p < 0.0001$. *Post-hoc* comparison with Tukey HSD further revealed how each gene contributed to significant sexual dimorphism: *Mus m. musculus*—*Mup*-B ($p = 0.002$), *Mup4* ($p = 0.0002$), *Obp7* ($p = 0.09$ ns); *Mus m. domesticus*—*Mup*-B ($p = 0.009$), *Mup4* ($p = 0.008$), *Obp7* ($p = 0.005$). The data and details of the model are provided in **Data Sheets 2**, **3**. Additionally, we did not detect any significant sex-differences in the expression of lipocalins between *M. m. musculus* and *M. m. domesticus*.

Biochemical Properties of Co-Expressed OBPs and MUPs

Our bioinformatics analysis revealed that OBPs and MUPs have different predicted isoelectric points with MUPs being more acidic than OBPs (2-tailed *t*-test, $p = 0.0009$; **Figure 4**), which differentially affects their solubility at different pH. Data are provided in **Data Sheet 4**.

Instead of looking at particular residues, we searched for a more general parameter that along with the structure directly affects lipocalin-binding properties. As a proxy, we calculated the grand average of hydropathy (i.e., GRAVY values, Xiong et al., 2009) which is defined as the sum of hydropathy values of all amino acids divided by the protein length. The values are negative for all individual members of MUPs and OBPs, however, the spectra of predicted hydrophobicities in OBPs and MUPs are non-overlapping (ANOVA, $F = 54.59$, $p < 0.0001$; Fligner-Killeen test of homogeneity of variances (chi-squared = 3.474, $df = 1$, p-value = 0.06234).

Furthermore, we have separated the group of MUPs based on previous studies (Logan et al., 2008; Mudge et al., 2008) into the ancestral group-A genes and the later duplicated group-B genes. Statistically significant differences in their mean values are graphically represented in **Figure 4** by non-overlapping confidence intervals. Furthermore, the group-B MUPs evolved hydropathic properties that are intermediate between the group-A MUPs and OBPs.

DISCUSSION

This study attempts to extend the current knowledge on genes for olfactory signals in feral mice by measuring selected mRNA expression across tissues, thus, revealing which other glands and tissues may be involved in chemical communication. We have corroborated that the group-B *Mup* transcripts show sexual dimorphisms in the liver in both subspecies (Stopková et al., 2007) whereby males excrete higher levels of *Mup*s than females. The most interesting result of the current study is a sexually dimorphic pattern of the highly expressed ancestral group-A *Mup4* and the female-biased *Obp7* in lacrimal glands. Tears containing MUP4 and OBP7 are presumably spread onto the fur during a course of facial self-grooming, thus contributing to body odor with hydrophobic ligands that these proteins may transport. Nasal MUP4, however, was suggested to play roles in sequestering pheromones and possibly transporting them to their receptors (Sharrow et al., 2002).

The exocrine roles of lacrimal lipocalins are supported by observations that facial areas elicited strong neuronal activity response in the accessory olfactory bulb (Luo et al., 2003). Similarly *Obp5, Obp6, Obp7* are also highly expressed in lacrimal glands (*Obp5* and *Obp7* originally annotated in inbred C57BL/6 as *Obp1a* and *Obp1b*). Recently, OBP proteins have been identified in tears of the laboratory mouse C57BL/6 (Karn and Laukaitis, 2015). In this study, *Obp* transcripts seem to be co-expressed in combination with other lipocalins (e.g., nasal and lacrimal *Obp5, Obp7, Mup4,* and *Lcn11*). OBP proteins (OBP5 and OBP7) were also predicted to form hetero-dimers (Pes et al., 1992) which may explain why *Obp5* and *Obp7* expression levels cluster together (**Figure 3**). Additionally, lacrimal expression of *Obp7* is female biased and thus, may have female specific roles in chemical communication.

Evaluation of mRNA distribution across tissues also revealed that some lipocalin genes are expressed in just one tissue. Similarly to the group-B *Mup* genes that are mostly expressed in the liver, *Obp6* is expressed only in lacrimal glands, and *Lcn4* is expressed almost exclusively in the vomeronasal organ where LCN4 protein is covering the vomeronasal sensory epithelium to enable primary reception of pheromones (Miyawaki et al., 1994). It is therefore likely, that LCN4 together with Vomeromodulin (Khew-Goodall et al., 1991) and MUP4 (Sharrow et al., 2002) participate in the process of pheromone access and detection by VNO. Following the process of pheromone detection, some of these proteins (LCN3, LCN4, VM, OBPs) are presumably transported to the oral cavity where they are often detected in saliva of C57BL/6 mice (Blanchard et al., 2015; Karn and Laukaitis, 2015) but, as we found in this study, their mRNAs are produced elsewhere (i.e., mainly VNO).

Obp and *Mup* (or *Lcn*) genes are co-expressed in particular tissues probably because their proteins have non-overlapping ligand-binding properties (Cavaggioni et al., 1990) with MUPs having higher and OBPs lower number of hydrophobic residues. This has been originally reported for two OBPs in inbred mice (Cavaggioni et al., 1987; Pes et al., 1992; Pelosi, 1994) and extended for newly detected OBPs in feral mice in this study. Therefore, we also suggest that co-expressed lipocalins may have complementary functions where MUPs may transport more hydrophobic volatiles to and from the vicinity of olfactory receptors whilst OBPs may transport less hydrophobic ligands or may play roles in the deactivation of partially degraded non-specific (i.e., less hydrophobic = hydroxylated or oxidized) volatiles after the signal transduction (Strotmann and Breer, 2011). Our analysis plots in **Figure 4** support such dual functionality. MUPs and OBPs have different pI and therefore may be active under different pH. We have already suggested that this difference in pI may imply that MUPs and OBPs have differential activities during cyclic (de-)

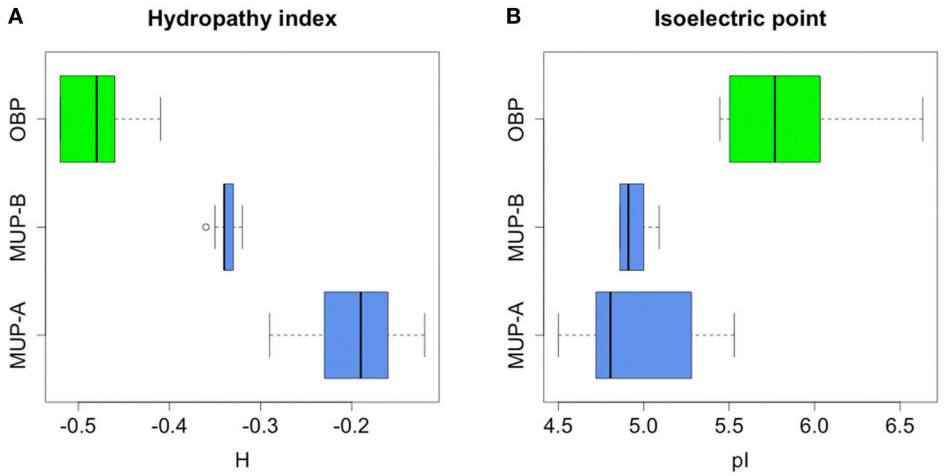


FIGURE 4 | Bioinformatic analysis of hydropathy properties of MUPs and OBPs. Group-A MUPs, and OBPs have non-overlapping hydropathy properties. Interestingly, excretory group-B MUPs adopted a position between more hydropathic group-A MUPs (i.e., with more hydrophobic beta barrel) and less hydrophobic OBPs **(A)**. Furthermore, MUPs have lower pI than OBPs **(B)**.

acidification of nasal mucosa during ventilation (Stopková et al., 2014) similarly as in the study by Cichy et al. (2015) who provided evidence that extracellular pH regulates excitability of vomeronasal sensory neurons. Also, the acidification balance is maintained by Carbonic anhydrase IV (CA IV) which is secreted by salivary, lacrimal, and nasal glands (Kimoto et al., 2004).

The importance of MUPs and OBPs for general olfaction has previously been reported by Sharrow et al. (2002) who analyzed binding properties of nasal *Mup4*, and by Utsumi et al. (1999) who provided evidence that the expression of nasal *Mup* (i.e., most likely *Mup5*) and *Obp* genes is high. Furthermore, many species do not have multiple copies of *Mup* genes and thus MUP products—a major component of chemical signaling and olfaction in mice and rats—but express functional OBPs. This has been shown in many mammals (Singer and Macrides, 1990; Stopkova et al., 2010; Hagemeyer et al., 2011; Nagnan-Le Meillour et al., 2014) and it is our hope that potentially diverse functions—i.e., including the detoxification roles (Stopková et al., 2009; Kwak et al., 2011, 2016)—of these proteins will be further resolved.

CONCLUSIONS

We have studied the expression of known and newly described mRNAs coding for nasal, lacrimal, salivary and urinary lipocalins that are characteristic for their unusually high quantities and the capacity to bind pheromones in their beta barrel. Many of these proteins were individually reported in previous studies by various authors. However, we have provided evidence that some proteins found in saliva are produced by multiple tissues with the normalized expression levels being as high or higher as those described for the urinary group-B *Mup* genes in the liver. For the first time, we have described a novel cluster of odorant binding proteins in feral mice and shown that some of them are differentially expressed in tissues or are sexually dimorphic. Some lipocalins (OBP, MUP, LCN) are co-expressed probably to widen the spectrum of potential ligands that these proteins may sequester and transport. Such expression pattern is almost identical in the two studied subspecies of the house mouse with the exception of VNO, which shows higher lipocalin expression in *M. m. musculus* males. Moreover, further study with sufficient sample sizes could further reveal the level of variation between different individuals and species.

AUTHOR CONTRIBUTIONS

RS performed qPCR analyses and wrote the manuscript. DV and JS were involved in transcript sequencing and qPCR analyses. BK did the bioinformatics analysis of OBP properties. OŠ and ZZ did the MS analyses. TA did most of the statistical analyses. KD was involved in all dissections and sample preparations. PS was involved in designing the experiment, in proteomic analyses and in writing the ms. All authors participated in writing the manuscript.

ACKNOWLEDGMENTS

The research of PS, RS, KD, DV, and JS was supported by the Grant Agency of the Czech Republic GACR No. P506/12/1046, and by the project "BIOCEV—Biotechnology and Biomedicine Centre of the Academy of Sciences and Charles University in Prague" (CZ.1.05/1.1.00/02.0109), from the European Regional Development Fund. BD was supported from a student grant SVV 260 313 / 2016 from Charles University in Prague. The MALDI-MS/MS part of the work (OŠ a ZZ) was carried out with the support of Proteomics Core Facility of CEITEC – Central European Institute of Technology, ID number CZ.1.05/1.1.00/02.0068, financed from European Regional Development Fund and by the Grant Agency of the Czech Republic GACR No. P206/12/G151.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fevo.2016.00047

Data Sheet 1 | Protein identification and destaining protocol.

Data Sheet 2 | Multiple sequence alignment of OBPs.

Data Sheet 3 | Original data for statistical analyses.

Data Sheet 4 | Details of the statistical model.

REFERENCES

Bignetti, E., Cavaggioni, A., Pelosi, P., Persaud, K. C., Sorbi, R. T., and Tirindelli, R. (1985). Purification and characterisation of an odorant-binding protein from cow nasal tissue. *Eur. J. Biochem.* 149, 227–231. doi: 10.1111/j.1432-1033.1985.tb08916.x

Blanchard, A. A., Ezzati, P., Shamshurin, D., Nistor, A. C., Leygue, E., Wilkins, J. A., et al. (2015). Towards further defining the proteome of mouse saliva. *Proteome Sci.* 13, 10. doi: 10.1186/s12953-015-0068-3

Buck, L., and Axel, R. (1991). A novell multigene family may encode odorant receptors: a molecular basis for odor recognition. *Cell* 65, 175–187. doi: 10.1016/0092-8674(91)90418-X

Cavaggioni, A., Findlay, J. B., and Tirindelli, R. (1990). Ligand binding characteristics of homologous rat and mouse urinary proteins and pyrazine-binding protein of calf. *Comp. Biochem. Physiol. B* 96, 513–520. doi: 10.1016/0305-0491(90)90049-y

Cavaggioni, A., Mucignat, C., and Tirindelli, R. (1999). Pheromone signalling in the mouse: role of urinary proteins and vomeronasal organ. *Arch. Ital. Biol.* 137, 193–200.

Cavaggioni, A., Sorbi, R. T., Keen, J. N., Pappin, D. J. C., and Findlay, J. B. C. (1987). Homology between the pyrazine-binding protein form nasal mucosa and major urinary proteins. *FEBS Lett.* 212, 225–228. doi: 10.1016/0014-5793(87)81349-2

Cichy, A., Ackels, T., Tsitoura, C., Kahan, A., Gronloh, N., Söchtig, M., et al. (2015). Extracellular pH regulates excitability of vomeronasal sensory neurons. *J. Neurosci.* 35, 4025–4039. doi: 10.1523/JNEUROSCI.2593-14.2015

Crawley, M. J. (2007). *The R Book*. Chichester: Wiley Publishing.

Cunningham, C. B., Nelson, A. C., Ruff, J. S., and Potts, W. K. (2013). MUP expression is linked with sociality not competitive ability in male house mice. *Integr. Comp. Biol.* 53, E46.

Dureje, L., Bímová-Voslajerova, B., and Piálek, J. (2011). No postnatal maternal effect on male aggressiveness in wild-derived strains of house mice. *Aggress. Behav.* 37, 48–55. doi: 10.1002/ab.20371

Felicioli, A., Ganni, M., Garibotti, M., and Pelosi, P. (1993). Multiple types and forms of odorant-binding proteins in the Old-World porcupine *Hystrix cristata*. *Comp. Biochem. Physiol. B* 105, 775–784. doi: 10.1016/0305-0491(93)90119-p

Flower, D. (1996). The lipocalin protein family: structure and function. *Biochem. J.* 318, 1–14. doi: 10.1042/bj3180001

Fluckinger, M., Haas, H., Merschak, P., Glasgow, B. J., and Redl, B. (2004). Human tear lipocalin exhibits antimicrobial activity by scavenging microbial siderophores. *Antimicrob. Agents Chemother.* 48, 3367–3372. doi: 10.1128/AAC.48.9.3367-3372.2004

Hagemeyer, P., Begall, S., Janotova, K., Todrank, J., Heth, G., Jedelsky, P. L., et al. (2011). Searching for major urinary proteins (MUPs) as chemosignals in urine of subterranean rodents. *J. Chem. Ecol.* 37, 687–694. doi: 10.1007/s10886-011-9971-y

Hiadlovska, Z., Bimova, B. V., Mikula, O., Pialek, J., and Macholan, M. (2013). Transgressive segregation in a behavioural trait? *Explorative strategies in two house mouse subspecies and their hybrids. Biol. J. Linnean Soc.* 108, 225–235. doi: 10.1111/j.1095-8312.2012.01997.x

Hurst, J. L., Payne, C. E., Nevison, C. M., Marie, A. D., Humphries, R. E., Robertson, D. H. L., et al. (2001). Individual recognition in mice mediated by major urinary proteins. *Nature* 414, 631–634. doi: 10.1038/414631a

Hurst, J. L., Robertson, D. H. L., Tolladay, U., and Beynon, R. J. (1998). Proteins in urine scent marks of male house mice extend the longevity of olfactory signals. *Anim. Behav.* 55, 1289–1297. doi: 10.1006/anbe.1997.0650

Janotová, K., and Stopka, P. (2009). Mechanisms of chemical communication: the role of major urinary proteins. *Folia Zool.* 58, 41–55.

Janotova, K., and Stopka, P. (2011). The level of major urinary proteins is socially regulated in wild *Mus musculus musculus*. *J. Chem. Ecol.* 37, 647–656. doi: 10.1007/s10886-011-9966-8

Jones, D. T., Taylor, W. R., and Thornton, J. M. (1992). The rapid generation of mutation data matrices from protein sequences. *Comput. Appl. Biosci.* 8, 275–282. doi: 10.1093/bioinformatics/8.3.275

Karn, R. C., and Laukaitis, C. M. (2011). Positive selection shaped the convergent evolution of independently expanded kallikrein subfamilies expressed in mouse and rat saliva proteomes. *PLoS ONE* 6:e20979. doi: 10.1371/journal.pone.0020979

Karn, R. C., and Laukaitis, C. M. (2012). The roles of gene duplication, gene conversion and positive selection in rodent Esp and Mup pheromone gene families with comparison to the Abp family. *PLoS ONE* 7:e47697. doi: 10.1371/journal.pone.0047697

Karn, R., and Laukaitis, C. (2015). Comparative proteomics of mouse tears and saliva: evidence from large protein families for functional adaptation. *Proteomes* 3, 283. doi: 10.3390/proteomes3030283

Khew-Goodall, Y., Grillo, M., Getchell, M. L., Danho, W., Getchell, T. V., and Margolis, F. L. (1991). Vomeromodulin, a putative pheromone transporter: cloning, characterization, and cellular localization of a novel glycoprotein of lateral nasal gland. *FASEB J.* 5, 2976–2982.

Kimoto, M., Iwai, S., Maeda, T., Yura, Y., Fernley, R. T., and Ogawa, Y. (2004). Carbonic anhydrase VI in the mouse nasal gland. *J. Histochem. Cytochem.* 52, 1057–1062. doi: 10.1369/jhc.3A6243.2004

Kiyono, H., and Fukuyama, S. (2004). NALT- versus Peyer's-patch-mediated mucosal immunity. *Nat. Rev. Immunol.* 4, 699–710. doi: 10.1038/nri1439

Kwak, J., Grigsby, C. C., Preti, G., Rizki, M. M., Yamazaki, K., and Beauchamp, G. K. (2013). Changes in volatile compounds of mouse urine as it ages: their interactions with water and urinary proteins. *Physiol. Behav.* 120, 211–219. doi: 10.1016/j.physbeh.2013.08.011

Kwak, J., Josue, J., Faranda, A., Opiekun, M. C., Preti, G., Osada, K., et al. (2011). Butylated hydroxytoluene is a ligand of urinary proteins derived from female mice. *Chem. Senses* 36, 443–452. doi: 10.1093/chemse/bjr015

Kwak, J., Strasser, E., Luzynski, K., Thoß, M., and Penn, D. J. (2016). Are MUPs a toxic waste disposal system? *PLoS ONE* 11:e0151474. doi: 10.1371/journal.pone.0151474

Lazar, J., Greenwood, D. R., Rasmussen, L. E. L., and Prestwich, G. D. (2002). Molecular and functional characterization of an odorant binding protein of the asian elephant, elephas maximus: implications for the role of lipocalins in mammalian olfaction. *Biochemistry* 41, 11786–11794. doi: 10.1021/bi0256734

Logan, D. W., Marton, T. F., and Stowers, L. (2008). Species specificity in major urinary proteins by parallel evolution. *PLoS ONE* 3:e3280. doi: 10.1371/journal.pone.0003280

Luo, M., Fee, M. S., and Katz, L. C. (2003). Encoding pheromonal signals in the accessory olfactory bulb of behaving mice. *Science* 299, 1196–1201. doi: 10.1126/science.1082133

Luo, M., and Katz, L. C. (2004). Encoding pheromonal signals in the mammalian vomeronasal system. *Curr. Opin. Neurobiol.* 2004, 428–434. doi: 10.1016/j.conb.2004.07.001

Marchese, S., Pes, D., Scaloni, A., Carbone, V., and Pelosi, P. (1998). Lipocalins of boar salivary glands binding odours and pheromones. *Eur. J. Biochem.* 252, 563–568. doi: 10.1046/j.1432-1327.1998.2520563.x

Maynard Smith, J., and Harper, D. D. (2003). *Animal Signals*. Oxford: Oxford University Press.

Miyawaki, A., Matsushita, F., Ryo, Y., and Mikoshiba, K. (1994). Possible pheromone-carrier function of two lipocalin proteins in the vomeronasal organ. *EMBO J.* 13, 5835–5842.

Moss, R. L., Flynn, R. E., Shen, X. M., Dudley, C., Shi, J., and Novotny, M. (1997). Urine-derived compound evokes membrane responses in mouse vomeronasal receptor neurons. *J. Neurophysiol.* 77, 2856–2862.

Mucignat-Caretta, C., Redaelli, M., Orsetti, A., Perriat-Sanguinet, M., Zagotto, G., and Ganem, G. (2010). Urinary volatile molecules vary in males of the 2 European subspecies of the house mouse and their hybrids. *Chem. Senses* 35, 647–654. doi: 10.1093/chemse/bjq049

Mudge, J. M., Armstrong, S. D., McLaren, K., Beynon, R. J., Hurst, J. L., Nicholson, C., et al. (2008). Dynamic instability of the major urinary protein gene family revealed by genomic and phenotypic comparisons between C57 and 129 strain mice. *Genome Biol.* 9, R91. doi: 10.1186/gb-2008-9-5-r91

Nagnan-Le Meillour, P., Vercoutter-Edouart, A. S., Hilliou, F., Le Danvic, C., and Lévy, F. (2014). Proteomic analysis of pig (*Sus scrofa*) olfactory soluble proteome reveals o-linked-n-acetylglucosaminylation of secreted odorant-binding proteins. *Front. Endocrinol.* 5:202. doi: 10.3389/fendo.2014.00202

Nelson, A. C., Cauceglia, J. W., Merkley, S. D., Youngson, N. A., Oler, A. J., Nelson, R. J., et al. (2013). Reintroducing domesticated wild mice to sociality induces adaptive transgenerational effects on MUP expression. *Proc. Natl. Acad. Sci. U.S.A.* 110, 19848–19853. doi: 10.1073/pnas.1310427110

Novotny, M. V. (2003). Pheromones, binding proteins and receptor responses in rodents. *Biochem. Soc.* 31, 117–122. doi: 10.1042/bst0310117

Novotny, M., Harvey, S., Jemiolo, B., and Alberts, J. (1985). Synthetic pheromones that promote inter-male aggression in mice. *Proc. Natl. Acad. Sci. U.S.A.* 82, 2059–2061. doi: 10.1073/pnas.82.7.2059

Pelosi, P. (1994). Odorant-binding proteins: structural aspects. *Crit. Rev. Biochem. Mol. Biol.* 29, 199–228. doi: 10.3109/10409239409086801

Pes, D., Dal Monte, M., Ganni, M., and Pelosi, P. (1992). Isolation of two odorant-binding proteins from mouse nasal tissue. *Comp. Biochem. Physiol.* 103B, 1011–1017. doi: 10.1016/0305-0491(92)90231-f

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 29, e45. doi: 10.1093/nar/29.9.e45

Phelan, M. M., McLean, L., Hurst, J. L., Beynon, R. J., and Lian, L. Y. (2014). Comparative study of the molecular variation between 'central' and 'peripheral' MUPs and significance for behavioural signalling. *Biochem. Soc. Trans.* 42, 866–872. doi: 10.1042/BST20140082

Roberts, S., C., Havlicek, J., Flegr, J., Hruskova, M., Littie, A. C., Jones, B. C., et al. (2004). Female factial attractiveness increases during the fertile phase of the menstrual cycle. *Proc. R. Soc. Lond. B* 271, S270–S272. doi: 10.1098/rsbl.2004.0174

Roberts, S. A., Davidson, A. J., McLean, L., Beynon, R. J., and Hurst, J. L. (2012). Pheromonal induction of spatial learning in mice. *Science* 338, 1462–1465. doi: 10.1126/science.1225638

Roberts, S. A., Simpson, D. M., Armstrong, S. D., Davidson, A. J., Robertson, D. H., McLean, L., et al. (2010). Darcin: a male pheromone that stimulates female memory and sexual attraction to an individual male's odour. *BMC Biol.* 8:75. doi: 10.1186/1741-7007-8-75

Shahan, K., Denaro, M., Gilmartin, M., Shi, Y., and Derman, E. (1987a). Expression of six mouse major urinary protein genes in the mammary, parotid, sublingual, submaxillary, and lachrymal glands and in the liver. *Mol. Cell. Biol.* 7, 1947–1954.

Shahan, K., and Derman, E. (1984). Tissue-specific expression of major urinary protein (MUP) genes in mice: characterization of MUP mRNAs by restriction mapping of cDNA and by *in vitro* translation. *Mol. Cell. Biol.* 4, 2259–2265. doi: 10.1128/MCB.4.11.2259

Shahan, K., Gilmartin, M., and Derman, E. (1987b). Nucleotide sequences of liver, lachrymal, and submaxillary gland mouse major urinary protein mRNAs: mosaic structure and construction of panels of gene-specific synthetic oligonucleotide probes. *Mol. Cell. Biol.* 7, 1938–1946.

Sharrow, S. D., Vaughn, J. L., Žídek, L., Novotny, M. V., and Stone, M. J. (2002). Pheromone binding by polymorphic mouse major urinary proteins. *Protein Sci.* 11, 2247–2256. doi: 10.1110/ps.0204202

Shaw, P. H., Held, W. A., and Hastie, N. D. (1983). The gene family for major urinary proteins: expression in several secretory tissues of the mouse. *Cell* 32, 755–761. doi: 10.1016/0092-8674(83)90061-2

Singer, A., and Macrides, F. (1990). Aphrodisin: pheromone or transucer? *Chem. Senses* 15, 199–203.

Smadja, C., and Ganem, G. (2002). Subspecies recognition in the house mouse: a study of two populations from the border of a hybrid zone. *Behav. Ecol.* 13, 312–320. doi: 10.1093/beheco/13.3.312

Smadja, C., and Ganem, G. (2008). Divergence of odorant signals within and between the two European subspecies of the house mouse. *Behav. Ecol.* 19, 223–230. doi: 10.1093/beheco/arm127

Spinelli, S., Ramoni, R., Grolli, S., Bonicel, J., Cambillau, C., and Tegoni, M. (1998). The structure of the monomeric porcine odorant binding protein sheds light on the domain swapping mechanism. *Biochemistry* 37, 7913–7918. doi: 10.1021/bi980179e

Stopka, P., Janotova, K., and Heyrovsky, D. (2007). The advertisement role of major urinary proteins in mice. *Physiol. Behav.* 91, 667–670. doi: 10.1016/j.physbeh.2007.03.030

Stopková, R., Hladovcová, D. J. K., Vyoral, D., and Stopka, P. (2009). Multiple roles of secretory lipocalins (MUP, OBP) in mice. *Folia Zool.* 58, 29–40.

Stopková, R., Stopka, P., Janotová, K., and Jedelský, P. L. (2007). Species-specific expression of major urinary proteins in the house mice (*Mus musculus musculus* and *Mus musculus domesticus*). *J. Chem. Ecol.* 33, 861–869. doi: 10.1007/s10886-007-9262-9

Stopková, R., Dudková, B., Hájková, P., and Stopka, P. (2014). Complementary roles of mouse lipocalins in chemical communication and immunity. *Biochem. Soc. Trans.* 42, 893–898. doi: 10.1042/BST20140053

Stopkova, R., Zdrahal, Z., Ryba, S., Šedo, O., Sandera, M., and Stopka, P. (2010). Novel OBP genes similar to hamster Aphrodisin in the bank vole, *Myodes glareolus*. *BMC Genomics* 11:45. doi: 10.1186/1471-21 64-11-45

Strotmann, J., and Breer, H. (2011). Internalization of odorant-binding proteins into the mouse olfactory epithelium. *Histochem. Cell Biol.* 136, 357–369. doi: 10.1007/s00418-011-0850-y

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* 28, 2731–2739. doi: 10.1093/molbev/msr121

Thoß, M., Luzynski, K., Ante, M., Miller, I., and Penn, D. J. (2015). Major urinary protein (MUP) profiles show dynamic changes rather than individual 'barcode' signatures. *Front. Evol.* 3:71. doi: 10.3389/fevo.2015. 00071

Thonhauser, K. E., Raveh, S., Hettyey, A., Beissmann, H., and Penn, D. J. (2013). Scent marking increases male reproductive success in wild house mice. *Anim. Behav.* 86, 1013–1021. doi: 10.1016/j.anbehav.2013. 09.004

Timm, D. E., Baker, L. J., Mueller, H., Zidek, L., and Novotny, M. V. (2001). Structural basis of pheromone binding to mouse major urinary protein (MUP-I). *Protein Sci.* 10, 997–1004. doi: 10.1110/ps.52201

Tirindelli, R., Mucignat-Caretta, C., and Ryba, N. J. P. (1998). Molecular aspects of pheromonal communication via the vomeronasal organ of mammals. *Trends Neurosci.* 21, 482–486. doi: 10.1016/S0166-2236(98)01274-0

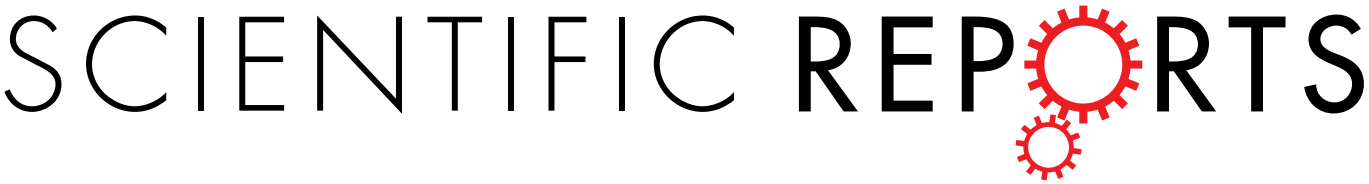
Utsumi, M., Ohno, K., Kawasaki, Y., Tamura, M., Kubo, T., and Tohyama, M. (1999). Expression of major urinary protein genes in the nasal glands associated with general olfaction. *J. Neurobiol.* 39, 227–236.

Venables, W. N., and Smith, D. M. (2009). *An Introduction to R: A Programming Environment for Data Analysis and Graphics, Version 2.9.0*. Bristol, CT: Network Theory.

Whitten, W. K., Bronson, F. H., and Greenstein, J. A. (1968). Estrus-inducing pheromone of male mice: transport by movement of air. *Science* 161, 584–585. doi: 10.1126/science.161.3841.584

Xiong, X., Huang, S., Zhang, H., Li, J., Shen, J., Xiong, J., et al. (2009). Enrichment and proteomic analysis of plasma membrane from rat dorsal root ganglions. *Proteome Sci.* 7, 41. doi: 10.1186/1477-5956-7-41

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.



OPEN

On the saliva proteome of the Eastern European house mouse (*Mus musculus musculus*) focusing on sexual signalling and immunity

Received: 27 April 2016
Accepted: 08 August 2016
Published: 31 August 2016

Pavel Stopka, Barbora Kuntová, Petr Klempt, Leona Havrdová, Martina Černá & Romana Stopková

Chemical communication is mediated by sex-biased signals abundantly present in the urine, saliva and tears. Because most studies concentrated on the urinary signals, we aimed to determine the saliva proteome in wild *Mus musculus musculus*, to extend the knowledge on potential roles of saliva in chemical communication. We performed the gel-free quantitative LC-MS/MS analyses of saliva and identified 633 proteins with 134 (21%) of them being sexually dimorphic. They include proteins that protect and transport volatile organic compounds in their beta barrel including LCN lipocalins, major urinary proteins (MUPs), and odorant binding proteins (OBPs). To our surprise, the saliva proteome contains one MUP that is female biased (MUP8) and the two protein pheromones MUP20 (or 'Darcin') and ESP1 in individuals of both sex. Thus, contrary to previous assumptions, our findings reveal that these proteins cannot function as male-unique signals. Our study also demonstrates that many olfactory proteins (e.g. LCNs, and OBPs) are not expressed by submandibular glands but are produced elsewhere–in nasal and lacrimal tissues, and potentially also in other oro-facial glands. We have also detected abundant proteins that are involved in wound healing, immune and non-immune responses to pathogens, thus corroborating that saliva has important protective roles.

The sequence of the mouse genome provided a tool to study blueprints for all RNAs and proteins in mice[1]. Progenitors of modern laboratory mice were hybrids among *Mus musculus domesticus*, *Mus musculus musculus* and other subspecies. Though, laboratory mice have been widely and successfully used as experimental organisms in studies of biomarkers of physiological states[2] and of human pathological conditions, they may be less suitable to study chemical communication, a process which is driven by sexual selection. This is due to the differential contribution of blocks of genes from the two house-mouse subspecies *M. m. domesticus* and *M. m. musculus* to current laboratory strains[1] that may mask typical intra- and inter-specific differences. One of our aims, therefore, was to define the saliva proteome in *M. m. musculus* to provide the array of proteins and their quantity characteristic for saliva in a wild living mouse species. This study also represents a baseline for future comparative studies focusing on chemical communication and immunity.

In mice, the most published studies in chemical communication focused on the major urinary proteins (MUPs)[3–7], which are expressed by the liver and transport volatile organic compounds (VOCs) in their beta barrel structure to the urine[6–11]. VOCs are slowly released from different urinary MUPs, and have been proposed to function in a variety of social signals, including identity, territorial marking, mate choice etc.[3,12–14]. Thus, lipocalins and their specific ligands together form a signal[15]. Differential ligand binding may have a potential influence on sub-species recognition between *M. m. musculus* and *M. m. domesticus*[16–18]. These two sub-species have been previously shown to vary in the abundance of male VOCs[19] and in MUP expression between the two subspecies and individuals of the opposite sex[9]. Moreover, scent signals have been shown to be an integral part of subspecies recognition and could play important roles in preventing interspecific mating between the two house mouse subspecies[18].

Increasing number of papers, however, show that MUP expression is linked to reproduction and sociality, and not just to competitive ability[3,4,20–23]. The expression of urinary MUPs is socially regulated in that males excrete

BIOCEV group, Department of Zoology, Faculty of Science, Charles University in Prague, Viničná 7, CZ 12844, Czech Republic. Correspondence and requests for materials should be addressed to P.S. (email: pstopka@natur.cuni.cz)

higher quantities of MUPs in contacts with females in the laboratory mouse[23] as well as in wild *M. m. musculus*[21], and in *M. m. domesticus*[3]. Furthermore, MUPs have a predictive value for the onset of aggressive behaviour and dispersal tendency in male wild house mice[24] and it is evident that scent marking signals have strong effects on the reproductive success of the signaller[20]. Furthermore, amongst MUPs, MUP20 (or 'Darcin') has been reported to predict the outcome of male-male territorial competition[3].

MUPs are products of a gene cluster that contains 21 coding genes (and a similar number of pseudogenes), and can be divided into two groups, the group-A (ancestral), containing *Mup3, Mup4, Mup5, Mup6, Mup20* and *Mup21*, and the group-B, consisting of 15 other *Mup*s (i.e. *Mup1, Mup2, Mup7-Mup19*) sharing almost 99% sequence identity[25,26], reviewed in refs 22,27. Different MUPs were originally supposed to present an individual 'barcode' signal[13] by which different individuals recognize each other. However, a recent study with a sufficient sample size shows that the urinary profiles of wild male house mice *M. m. musculus* are not individually unique but are dynamic over time with significant changes after puberty and during adulthood[28]. Moreover, the variation in pheromone affinities of the urinary MUP isoforms provides low support for the proposal that heterogeneity in MUPs plays a role in regulating profiles of available pheromones[10]. However, MUPs have also been reported in several tissues other than the liver including salivary glands, olfactory/vomeronasal epithelia, and nasal-associated lymphoid tissues[2,8,29–31], but their functions are not yet fully understood.

Another interesting group of lipocalins involved in chemical communication are products of the odorant binding protein genes (*Obp*). The X-linked *Obp* genes were thought to involve just two nasal members–*Obp1a*, and *Obp1b*[32]. However, *Obp* genes have undergone a series of duplications in mice, and they occur in a cluster of seven genes (i.e. including *Prb*, Probasin) and two pseudogenes on the X chromosome[27,31,33]. All OBPs including Probasin have a specific disulfide bond (Cys38–Cys42), which represents a strong OBP-diagnostic motif CXXXC - Cys-Xaa-Xaa-Xaa-Cys[27,33]. To date, *Obp*s/OBPs were detected in various mammalian taxa, e.g., house mice[31], bank voles[34], porcupines[35], elephants[36], cows[37], and boar[38,39]. Interestingly, pigs have OBPs and SAL. SAL is the major salivary protein in pigs with affinity to steroids and to 2-isobutyl-3-methoxypyrazine, it is phylogenetically close to MUPs and is expressed by the male submaxillary glands[40]. Furthermore, aphrodisin is an OBP[34] described as the major pheromone transporter in vaginal flushes of hamsters (*Cricetus cricetus*)[41].

In our latest study[31], we described particular mRNA expression sites for the newly described odorant binding proteins in wild mice (*M. m. musculus, M. m. domesticus*). They are highly expressed with other lipocalins (LCNs, MUPs) in the mouse lacrimal, nasal, and vomeronasal tissues with the normalized expression levels being as high or higher as those described for the urinary group-B *Mup* genes in the liver. Lacrimal glands expressed the mRNA coding OBP5, OBP6 and OBP7 whilst the mRNAs coding OBP1/OBP2, OBP5, and OBP7 were highly abundant in the olfactory epithelia (OE), vomeronasal organ (VNO), and nasal-associated lymphoid tissue (NALT) in the both house mouse subspecies. No *Obp* mRNA was detected in submandibular glands but at least one OBP protein (i.e. OBP5) was detected in the saliva. We have also provided evidence that *Obp* transcripts are co-expressed in combination with other lipocalin transcripts (e.g. nasal and lacrimal *Obp5* and *Obp7* with *Mup4* and *Lcn11*), presumably to widen the spectrum of ligands that OBP, MUP, and LCN proteins may sequester and transport[31]. Thus, this study also aims to detect particular OBPs in the mouse saliva to test whether OBPs are involved in the transport of VOCs and various degradation products from the nasal and lacrimal tissues to the oral cavity where digestion starts.

This study was conducted to detect the level of sexual dimorphisms with a particular interest in lipocalins that have potential roles in chemical communication as transporters of salivary VOCs. We used sensitive proteomic techniques to identify proteins and partially also RNAseq on GS Junior to detect a potential expression site for some of the lipocalins of particular interest (i.e. MUP20, OBPs). Because eyes, nose and mouth are primary gates for various pathogens to enter the body and at the same time a route of receiving or transmitting pheromones, it is believed that an organismal detoxification, immunity and chemical communication might have been driven by similar evolutionary forces[27,33,42]. Thus, we also discuss our results on other protein families significantly enriched in the saliva proteome of the house mouse.

Materials and Methods

Ethical Standards. All animal procedures were carried out in strict accordance with the law of the Czech Republic paragraph 17 no. 246/1992 and the local ethics committee of the Faculty of Science, Charles University in Prague specifically approved this study in accordance with the accreditation no. 27335/2013-17214 valid through 2019.

Animals. To allow for natural variation between samples we selected individuals of similar weight, in reproductive condition, but from different sites: 1M+1F locality Bříza (50.3605111N, 14.2162558E), 1M+3F Velke Bilovice (48.8492886N, 16.8922736E), 3M Bohnice (50.1341539N, 14.4142189E), 1F Dolni Brezany (49.9632106N, 14.4585047E), 1F Bruntal (49.9884447N, 17.4647019E). After the protein sample collection, all experimental individuals were sacrificed by cervical dislocation and tissues were further used for the transcriptome analyses.

Samples. The saliva samples were collected by gentle flushing with a pipette using 50 μl of the 0.9% saline solution from six female and five male biological replicates, and each sample was analysed twice to produce the mean values from the methodology duplicates. This was done in the 'in-house' Mass Spectrometry and Proteomics Service Laboratory, Faculty of Science, Charles University in Prague.

Protein Digestion. The protein concentration of each lysate was determined using the BCA assay kit (Fisher Scientific). Cysteins in 200 μg of proteins were reduced with the final concentration of 5 mM TCEP (60 °C for

60 min) and blocked with10 mM (10 min Room Temperature). Samples were cleaved with trypsine (i.e. 1/100, trypsine/protein). Peptides were desalted on Michrom C18 column.

nLC-MS^2 Analysis. Nano Reversed phase columns were used (EASY-Spray column, 50 cm × 75 μm ID, PepMap C18, 2 μm particles, 100 Å pore size). Mobile phase buffer A was composed of water, 2% acetonitrile and 0.1% formic acid. Mobile phase B contained 80% acetonitrile, and 0.1% formic acid. Samples were loaded onto the trap column (Acclaim PepMap300, C18, 5 μm, 300 Å Wide Pore, 300 μm × 5 mm, 5 Cartridges) for 4 min at 15 μl/min loading buffer was composed of water, 2% acetonitrile and 0.1% trifluoroacetic acid. After 4 minutes ventile was switched and Mobile phase B increased from 2% to 40% B at 60 min, 90% B at 61 min, hold for 8 minutes, and 2% B at 70 min, hold for 15 minutes until the end of run.

Eluting peptide cations were converted to gas-phase ions by electrospray ionization and analysed on a Thermo Orbitrap Fusion (Q-OT-qIT, Thermo). Survey scans of peptide precursors from 400 to 1600 m/z were performed at 120 K resolution (at 200 m/z) with a 5×10^5 ion count target. Tandem MS was performed by isolation at 1.5 Th with the quadrupole, HCD fragmentation with normalized collision energy of 30, and rapid scan MS analysis in the ion trap. The MS^2 ion count target was set to 10^4 and the max injection time was 35 ms. Only those precursors with charge state 2–6 were sampled for MS^2. The dynamic exclusion duration was set to 45 s with a 10 ppm tolerance around the selected precursor and its isotopes. Monoisotopic precursor selection was turned on. The instrument was run in top speed mode with 2 s cycles.

Data analysis. All data were analysed and quantified with the MaxQuant software (version 1.5.3.8)[43]. The false discovery rate (FDR) was set to 1% for both proteins and peptides and we specified a minimum peptide length to seven amino acids. The Andromeda search engine was used for the MS/MS spectra search against the Uniprot *Mus musculus* database (downloaded on June, 2015, containing 44,900 entries. Enzyme specificity was set as C-terminal to Arg and Lys, also allowing cleavage at proline bonds and a maximum of two missed cleavages. Dithiomethylation of cysteine was selected as fixed modification and N-terminal protein acetylation and methionine oxidation as variable modifications.

The "match between runs" feature of MaxQuant was used to transfer identifications to other LC-MS/MS runs based on their masses and retention time (maximum deviation 0.7 min) and this was also used in all quantification experiments. Quantifications were performed with the label-free algorithms described recently[43].

Gene ontology analysis. We used the PANTHER (Protein ANalysis THrough Evolutionary Relationships) Classification System to classify proteins according to Biological process, which is the function of the protein in the context of a larger network of proteins that interact to accomplish a process at the level of the tissue[44]. Each analysis involved Bonferroni corrections for multiple testing.

RNAseq. The submandibular glands were homogenized in RLT buffer (Qiagen) with MagNALyser (Roche) for 30 s at 6000 rpm. We used the RNeasy Mini Kit (Qiagen) for RNA isolation following the manufactures protocol with on-column DNase I treatment. The purity and concentration of eluted RNA was measured with NanoDrop ND1000. The quality of RNA was checked using the agarose gel electrophoresis and pre-selected samples were further analysed with Agilent Bioanalyzer using the RNA Nano 6000 chip to obtain information on the RNA integrity. Treated samples were cleaned using the RNA cleanup procedure (RNeasy Mini Kit) and checks of the quality with AGE and Bioanalyzer were repeated as well as the measurement with NanoDrop. RIN of twelve submandibular gland samples after this procedures ranged from 4.60 to 7.6. We decided to eliminate the worst two samples (i.e. one from female with RIN 4.60, and one from male with RIN 6.00). The remaining ten RNA samples RIN values were following: 4.70, 5.90, 7.00, 6.70, 5.90 for females and 6.40, 6.70, 7.60, 7.30 and 7.10 for males. RNA samples were standardly stored at −80 °C.

cDNA was prepared using the SMARTer PCR cDNA Synthesis Kit (Clontech) and amplified with Advantage 2 PCR Kit (Clontech). Both procedures were handled according to the Trimmer-2 Normalization Kit (Evrogen) protocol. The products of optimalized cDNA amplification were then loaded on AGE. For each sample, only the area of product in range from ~500 bp to ~1300 bp (well visible area full of bands) was excized from the gel and the DNA products were extracted using the Gel/PCR DNA Fragments Extraction Kit (Geneaid). To avoid potential contaminats contaminants we performed AMPure XP cleanup (Beckman-Coulter). Purified products (and the range where they emerge) were checked on AGE. DNA concentration was determined using Quant-it Pico Green dsDNA Assay Kit (Invitrogen) and fluorimeter (Hoefer DQ 300). For each obtained submandibular gland size-selected transcriptomes from 5 males and 5 females we prepared Rapid Libraries (RL) according to Rapid Library Preparation Manual (my454.com). Rapid libraries were checked for the presence of small fragments on BioAnalyzer using the High Sensitivity DNA kit. Equal amount from each of 10 Rapid Libraries (107 molecules per μl dilution) were mixed and then used for emPCR. Further steps were following the provider's instructions for sequencing with GS Junior+ (Roche; emPCR Amplification Method Manual Lib-L and Sequencing Method Manual, my454.com). In order to reach better sequencing depth we combined two sequencing runs (140 000 HQ reads and 120 000 HQ reads). Both.sff datasets were merged using the sff file tool (part of GS Junior+ Roche system software). Merged reads were then multiplexed, trimmed (i.e. using trimming database that contains primers used for the libraries preparation), filtered and aligned into contigs against *Mus musculus* cDNA database ("the super-set of all known, novel and pseudo gene predictions"; ensembl.org) and using GS Reference Mapper (Roche). Highly detected transcripts are graphically represented in Supplementary Figure 1.

Phylogenetic analysis by Maximum Likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model[45]. The tree with the highest log likelihood (−1578.6730) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying

Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 24 nucleotide sequences from the mouse genome. Codon positions included were 1st+2nd+3rd+Noncoding. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. Evolutionary analyses were conducted in MEGA5[46]. We followed the standard MGI/NCBI nomenclature for all proteins with the exception of OBPs where our recently[31] submitted *M. m. musculus* names/synonyms were used (i.e. *Obp1* - KJ605390, *Obp2* - KJ605391, *Obp5* - KJ605392, *Obp6* - KJ605393, and *Obp7* - KJ605394) instead of the old names originally provided for all *Obp* predicted transcripts and proteins of the laboratory mouse.

Protein structures. The protein PDBs were downloaded from RCSB Protein Data Bank (http://www.rcsb.org/) and visualized in a molecular visualization system PyMOL software v.1.5. (http://pymol.org). Examples of immunity-related proteins in the Results section are visualized on human homologs i.e. Cathelin-like domain of human Cathelicidin LL-37 (4EYC), Cystatin D (1ROA) and KLK1 (1SPJ) because no mouse structures exist to date. The last structure is an example of murine ESP1 (2LMK).

Data availability. Proteomic data from LC-MS/MS are provided publically available in Supplementary Data file 1. RNAseq data from GS Junior are provided as Supplementary Data file 2.

Results

The saliva proteome. We have generated the saliva proteome from the house mouse, *M. m. musculus* and detected a total of 633 proteins at 0.01 FDR (i.e. False Discovery Rate for all peptides and proteins). Successful identifications of these proteins resulted from a relatively high number of peptides per identification (11.33 ± 11.30, mean $\pm$ sd), sequence coverage ($35.1 \pm 20.2\%$), and unique sequence coverage (28.6 ± 17.8), Fig. 1a. Moreover, Spearman's rank correlation between coverage and unique peptide coverage was high and significant rho = 0.74, S = 10866000, p-value < 2.2e-16 as well as the correlation between the number of MS/MS spectra and coverage (rho = 0.71, S = 12230000, p-value < 2.2e-16). Thus, the most abundant proteins have higher coverage and unique peptide coverage than those that were less abundant.

Having produced the saliva proteome we next performed the analysis of differentially abundant proteins between males and females using the Power Law Global Error Model (PLGEM)[47]. First of all, we calculated the signal-to-noise ratio - STN, because it explicitly takes unequal variances into account and because it penalizes proteins that have higher variance in each class more than those proteins that have a high variance in one class and a low variance in another[47]. Because PLGEM can only be fitted on a set of replicates of a same experimental condition we have done this for female data, Fig. 1b. Correlation between the mean values and standard deviations was high ($r^2 = 0.98$, Pearson = 0.95) so we continued with the resampled STNs and calculated differences with corresponding p-values between males and females. We used the MA plot as a method of showing sex differences where fold differences are plotted against the base mean. Significant differentially abundant proteins are colored from green ($p < 0.05$) to blue ($p < 0.01$) in Fig. 1c.

PLGEM analysis of the level of sexual dimorphism revealed that 132 (21%) out of 633 identified proteins at 1% FDR and $p < 0.05$ were sexually dimorphic. Male biased proteins included 92 (14.5%) and female biased proteins included 40 (6.3%) successful identifications. Thus, the male-biased proteins were more common than the female-biased proteins in the saliva proteome of the house mouse subspecies *M. m. musculus*.

The most abundant salivary proteins. Based on the median value we sorted our data to detect the most abundant proteins in the saliva proteome. We have filtered out potential contaminants such as keratins and also trypsins which are the enzymes that cleave all peptides before LC-MS in this study. The top five percent of the most abundant proteins included (i.e. in descending order): MUP6, BPIFB9B, ALBU, OBP5, OBP7, MUP5, CAH6, SCGB2B2, OBP1, ADA, SCGB1B2, AMY1, ACTB, PIP, LCN11, LACREIN, SCGB1B27, LCN13, KLK1KB9, OVOS, OBP2 (lipocalins are underlined). Out of these top abundant proteins, a third (i.e. seven proteins) was sex biased with male-biased OBP1, OBP2, LCN13, BPIFB9B, OVOS, AMY1 and female-biased KLK1B9.

Lipocalins and other proteins involved in chemical communication. One of the most interesting results in our study is a finding that the saliva proteome is very rich of lipocalins belonging to different lipocalin sub-families and originating from several oro-facial expression sites. We have detected 20 (out of 55) mouse lipocalins belonging to the well annotated groups of LCNs (LCN2, LCN3, LCN4, LCN11, LCN12, LCN13, LCN14), OBPs (OBP1, OBP2, OBP5, OBP6, OBP7)[31,33], and MUPs (MUP4, MUP5, MUP6, MUP8, MUP14, MUP17, MUP20, MUP21)[26]. A total of 10 lipocalins (50%) was significantly ($p < 0.05$) sexually dimorphic (OBP1, OBP2, LCN3, LCN4, LCN13, LCN14, MUP4, MUP8, MUP14, and MUP20). Only MUP8 was female biased ($p = 0.026$), while all other sexually dimorphic lipocalins were male biased. Furthermore, we have detected MUPs from the both earlier described phylogenetic groups (i.e. the ancestral group-A MUPs, and the later evolved group-B MUPs, refs 25,26) in the saliva. Moreover, OBPs and MUPs each belong to monophyletic groups of genes (bootstrap > 75) whilst LCNs are more heterogeneous and only LCN3, LCN4, LCN13, LCN14 form a monophyletic group previously detected in the mouse nasal and vomeronasal tissues. The complete mouse lipocalin phylogeny is provided elsewhere[27,33].

The abundance of the male-biased MUP20 ($p = 0.017$) in the saliva was unexpected (Diagnostic peptides: FAQLSEEHGIVR, ENIIDLTNANR) because MUP20 has previously been detected only in the urine of male *Mus musculus domesticus* and C57BL/6. Therefore, we performed RNAseq-based analysis of the submandibular gland transcriptome to support this identification. We have detected the mRNA expression of several *Mup* genes

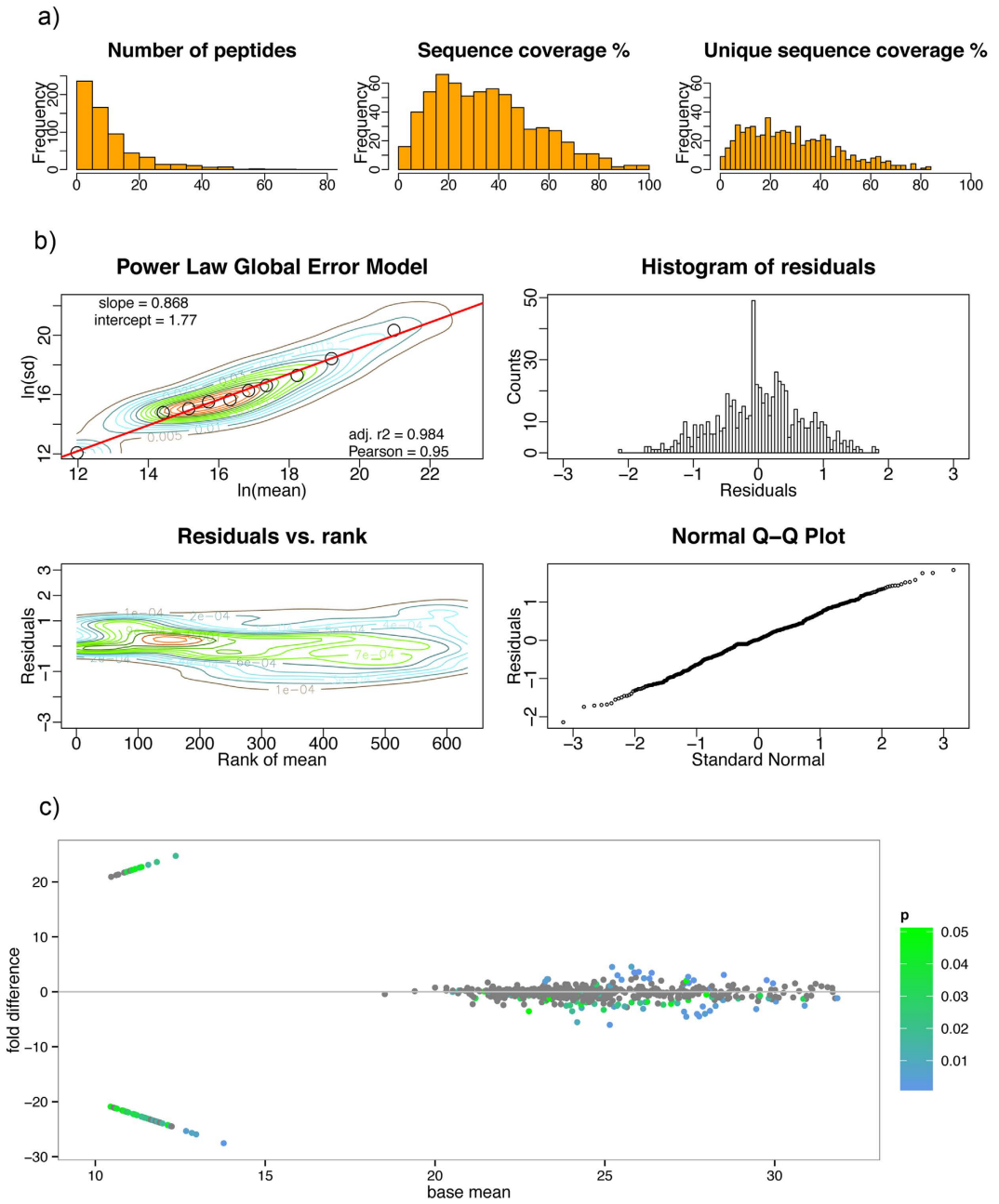


Figure 1. Details of the Power Law Global Error Model[47]: (**a**) histograms of the sequence coverage and the unique sequence coverage, (**b**) the model fitting on a female experimental condition, (**c**) MA plot with the differentially abundant proteins where significant points are colored from green ($p < 0.05$) to blue ($p < 0.01$).

including *Mup20, Mup4, Mup5,* and *Mup9*. However, all *Obp* members were absent in SMG transcriptome which supports our previous observations that OBPs are mainly expressed by the nasal (OE, VNO, NALT) and lacrimal glands/tissues[31], and/or other as yet understudied oro-facial mouse glands. Similarly, male biased lipocalins LCN3 (VNSP1), LCN4 (VNSP2), LCN13 (OBP2A), LCN14 (OBP2B) are encoded by *Lcn* genes expressed by the nasal and vomeronasal tissues, but we detected them being highly abundant in the saliva proteome but not in the SMG transcriptome.

Along with lipocalins, we have also detected two male-biased exocrine glans-secreted peptides ESP1 ($p = 0.006$) and marginally ESP6 ($p = 0.08$), putative protein pheromones that are abundant in tears along with as yet uncharacterized lacrimal protein–Lacrein–which is also present in the saliva proteome of males and females but not in the transcriptome (SMG) in this study. Furthermore, we have also detected male-biased vomeromodulin (VOME, $p = 0.003$) in the saliva, however its expression site is also known to be exclusively the mouse vomeronasal organ[48].

Secretoglobins. In the saliva proteome of the mouse, we have detected 13 secretoglobin members with 7 of them being sexually dimorphic (i.e. male-biased: SCGB1B20, SCGB1B3, SCGB2A2, SCGB2B20, SCGB2B24, SCGB2B3, SCGB2B7) at $p < 0.05$. None of the salivary SCGB members was either male or female unique. On the level of SMG transcriptome, we have detected the expression of mRNA coding only three secretoglobins SCGB1B27, SCGB2B26, and SCGB2B27. The most abundant secretoglobin in the saliva proteome was a secretoglobin from family 2B, member 2 or SCGB2B2. However, we did not detect the mRNA coding SCGB2B2 in our trasncriptomic (SMG) data, thus, suggesting that SCGB2B2 (=ABPBG2) is also transported to the oral cavity from other tissues–most likely from the lacrimal or nasal glands/tissues[49].

Kallikreins and wound healing. Kallikreins are a group of serine proteases, which are capable of cleaving peptide bonds in various proteins also including some kallikreins. They have an antimicrobial activity and are involved in wound healing[50]. We have detected 4 kallikreins (KLK1, KLK10, KLK13, KLK14) and 13 kallikrein 1-related peptidases in saliva (KLK1B11, KLK1B16, KLK1B3, KLK1B1, KLK1B21, KLK1B22, KLK1B24, KLK1B26, KLK1B27, KLK1B4, KLK1B5, KLK1B8, KLK1B9). Kallikrein 1 and all kallikrein 1-related peptidases form a monophyletic cluster and it is notable in Fig. 2 that Kallikrein 1 is not an outgroup (i.e. the ancestral gene) to all other Kallikrein 1-related peptidases. Kallikreins KLK1, KLK10, and KLK14 were not sexually dimorphic whilst KLK13 was female biased but only marginally significant ($p = 0.054$) because it was detected only in three females. Almost all kallikrein 1-related peptidases were female biased ($p < 0.01$), except KLK1B5 ($p = 0.08$) that only revealed a trend. On the level of SMG transcriptome we have detected KLK1 and all Kallikrein 1-related peptidases. Furthermore, we have also detected angiotensinogen (ANGT) a substrate for KLK1 activity and CRAMP (i.e. Cathelicidin related anti-microbial peptide)–an antimicrobial peptide which is regulated by Kallikreins 5 and 7[50].

Proteins involved in innate immunity. Based on the functional classification and gene ontology, we have selected those genes that match our criteria, thus limiting the function to two keywords–immunity and antimicrobial. We have detected a total of 56 proteins fitting our criteria with 21 of them being significantly sexually dimorphic, Fig. 2. Additionally, we have identified 9 annexins equally expressed by individuals of both sex and which have strong effect upon the mechanism by which glucocorticoids (such as cortisol) inhibit inflammation.

Levels of sexual dimorphism are graphically represented in Fig. 2 with the full protein list provided in Data set 1. Interestingly, the immunity heat map in Fig. 2 shows rather low levels of the immunity-linked protein abundances except the three highly expressed 'bactericidal permeability-increasing proteins' (BPIB1, BPIFA2, and BPIFB9B) and one immunoglobulin (IGKC, Ig kappa chain C region). BPI proteins have an antibacterial activity against the gram-negative bacteria[51]. We have detected seven BPI members and all of them were male biased ($p < 0.05$): BPIA1, BPIB1, BPIB2, BPIB3, BPIFA2, BPIFB5, BPIFB9B. On the level of the mouse SMG transcriptome, however, we have detected only *Bpifa2* which has previously been detected as a transcript in the mouse parotid glands, and is also known as the parotid secretory protein - PSP[52]. Furthermore, BPIFA1 (PLUNC/SPLUNC1) and BPIFB1 (LPLUNC1) are known to be expressed by Bowman's glands of the nasal passage[53]. Remaining members were most likely expressed by other oro-facial tissues (i.e. nasal, lacrimal, palatal, and salivary). Moreover, we have also detected CRAMP, a cathelin-related antimicrobial peptide, in the saliva of males and females.

Discussion

With the use of sensitive proteomic techniques, we show that saliva is a complex system containing chemical signal transporters, antibacterial and immunity linked proteins, and many other proteins that are involved in general physiology of the oral cavity. We also show that many nasal and lacrimal proteins are abundant in the saliva proteome, presumably as a consequence of their final transport to the oral cavity from tissues where they are expressed and where they function as VOC transporters. These include a group of odorant binding proteins (OBP) that we previously identified as predicted transcripts in the mouse genome[27,33], detected their expression sites in various oro-facial tissues[31], and finally detected them as proteins in the saliva proteome in this study. Because OBPs, MUPs and LCNs have similar tertiary structure (Fig. 3) with the capacity to transport VOCs, it is likely that together, nasal lipocalins, could be important for signal transduction but even more for a consequent neuronal desensitisation by transporting partially degraded VOCs to the oral cavity and then further to the digestive tract. However, it is in question why OBP1 and OBP2 are sexually dimorphic. It is possible that different levels of expression may reflect potential differences in the olfactory abilities between sexes. To further support the claim that these proteins originate only in the nasal and lacrimal tissues, it would help to analyse other independent oro-facial glands (i.e. parotid, sublingual, or von Ebner glands) and lymphoid tissues that are present in the oral cavity.

Because mice begin social interactions by investigating facial areas[54] it is also likely that salivary proteins expressed by salivary glands along with those that were transported from the nose serve chemical communication together. It is however in question how would an individual benefit from having lipocalins with ligands that were inhaled from another individual. We suggest that a mixture of the self and of the other individual's smell, that is spread on the receiver's body during selfgrooming, could mediate peaceful social contacts between individuals within a deme–a structure typical for the house mouse social groups[55].

Among MUPs, MUP20 or 'Darcin' was previously described as a protein pheromone that stimulates female attraction for particular *M. m. domesticus* males, improves spatial learning[56,57], has been shown to function as an indicator of current health status of males[58], and to predict the outcome of male-male territorial competition[3]. MUP20 expression levels are higher in dominant males during and prior to competition, making it predictive of dominance status[3]. However, we detected MUP20 being significantly male-biased ($p = 0.017$) but abundant in the saliva proteomes in individuals of both sex in *M. m. musculus*, and our RNAseq data revealed that MUP20 is coded by *Mup20* gene in the mouse submandibular gland. Thus, it is hard to imagine that this protein functions

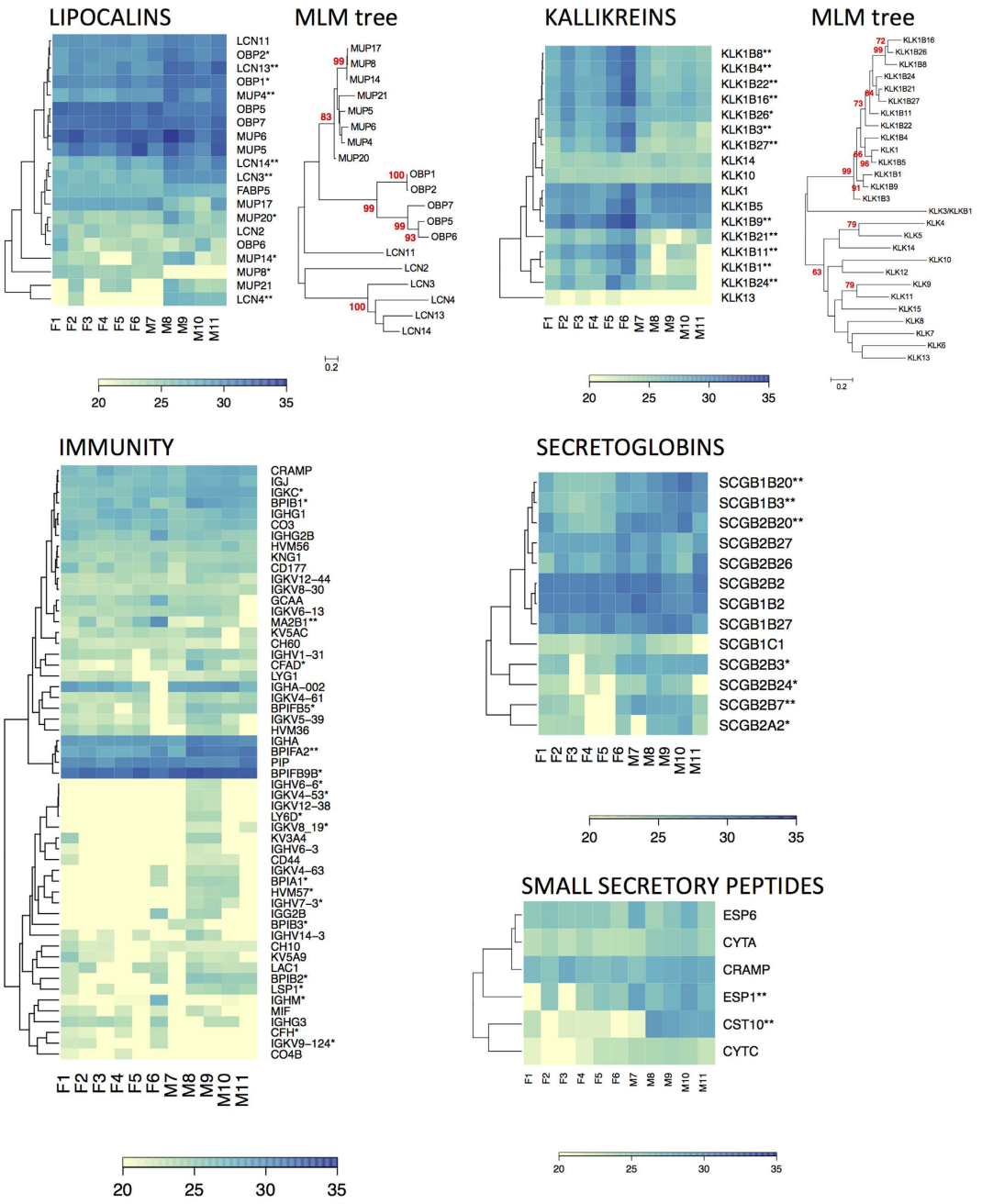


Figure 2. Graphical representation of the protein abundance values in heat maps shows sexually dimorphic proteins (labelled with stars: *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001) with notable variation between individuals. We provide the phylogeny dendrogram for kallikreins and a partial dendrogram for the detected lipocalins. The Maximum likelihood trees are showing the protein phylogeny based on the number of substitutions per site and with the bootstrap values. We consider a group of proteins monophyletic when bootstrap values are above 75.

as a male-only pheromone (i.e. at least in this subspecies) if females produce such signal too. There is at least one study demonstrating that MUP20 is also present in the submandibular gland transcriptome of the laboratory mouse[25]. Therefore, previous studies describing MUP20 as a male-specific signalling protein that is present only in the urine of *M. m. domesticus*[56,57] need to be further supported with more sensitive techniques. To add, we are also showing that MUP8 is among all MUPs the only one that is significant female biased. However, it remains to be determined where it is transcribed and translated.

Another protein that has been described as a male-specific signalling protein in the laboratory mouse is a 7 kDa protein, named as the exocrine gland-secreted peptide-1 or ESP1[59,60]. ESP1 is produced by the mouse lacrimal glands, secreted with tears and when experimentally transferred to the female vomeronasal organ, it stimulates V2R-expressing vomeronasal chemosensory neurons, and thus elicits an electrical response[60]. In *M. m. musculus* under this study, however, ESP1 and ESP6 were sex biased with the female levels being lower than those detected in males (Fig. 2). If ESPs cannot function as pheromones due to their occurrence in individuals of

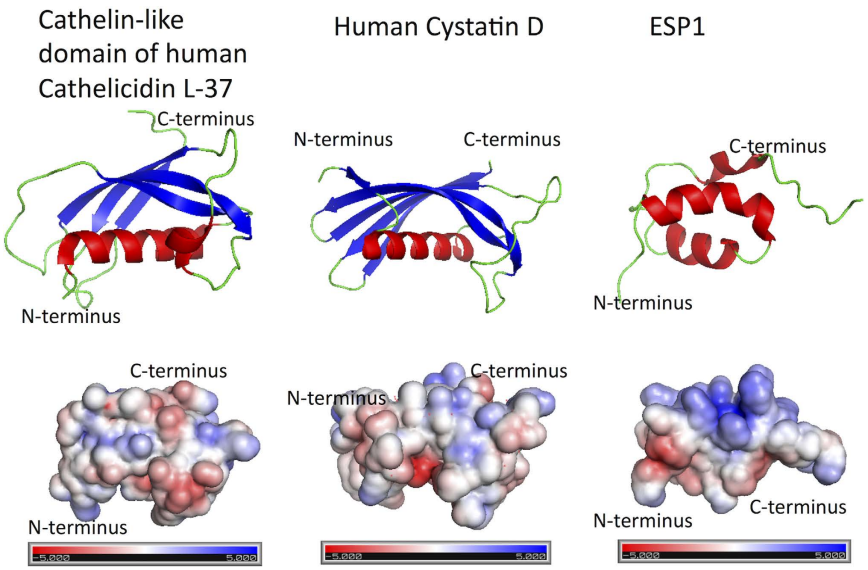


Figure 3. Graphical representation of structural and biochemical properties of murine salivary protein homologs - Cathelin-like domain of human Cathelicidin L-77, human Cystatin D and murine ESP1. Upper row: Cartoon representation of selected proteins where α-helices are in red, and beta sheets in blue color. Below are provided 3D representations of surface charge distribution of respective proteins, the charge scale is shown under each structure.

both sex, it is in question what are their other potential roles. One obvious role stems out of visualizing the electrostatics properties of ESP1 in Fig. 3. ESP1 has three α-helices with two helices being negatively charged and one (middle) being positively charged. This structural amphypathy fits the description of antimicrobial peptides (i.e. similar to CRAMP[61]). Thus it is possible that ESP1 and also other ESPs are involved in the host-defence against bacteria.

Secretoglobins (SCGBs or ABPs) were also suggested to play roles in chemical communication[62]. However, when experimentally tested, wild mice of the two subspecies did not show any difference in time spent sniffing urine to sniffing the urine with added ABPs[18]. In our data, some SCGBs/ABPs were male-biased but no member of this family was sex unique in the mouse saliva. Secretoglobins were previously detected in most body fluids and mucosa including lungs, uterus, nasal and oral cavities, and tears in many mammals including rabbits, mice, and humans. They are presumably involved in various processes including tissue repair, eye protection, and anti-inflammatory responses due to their capacity to transport various steroids[63]. Moreover, the PANTHER Overrepresentation test in this study did not identify any involvement in any known biological process of the mouse, which makes this family functionally understudied though an interesting system for future studies.

The saliva proteome also contains proteins that are involved in the regulation of harmonious symbiosis with bacteria and of potential risk of exogenous bacterial infection. A strategy called "nutritional immunity" prevents pathogens from acquiring the host iron[64], which is an essential nutrient, but only small amounts of free iron are accessible. Therefore, bacteria acquire iron by a secretion of high-affinity iron sequestrating siderophores. The mammalian host, however, limits this process by the production of Lipocalin 2 (LCN2)[65] which efficiently scavenges for catecholate-type siderophores (i.e. such as enterochelin, mycobactin)[66]. In our data the production of LCN2 (and also LCN11) was equal between sexes, thus suggesting that males and females similarly regulate such symbiosis with pathogens and/or the defence against them. Interestingly, when the lipocalin-acquired iron is transported to the oral cavity, which is the beginning of the digestive tract, the complex is in fact running towards the enzymatic digestion and thus iron is freed, and can presumably be used by symbiotic bacteria in the lower digestive tract. However, mammalian hosts evolved almost an array of other mechanisms of defence.

Other mechanisms of defence involve bactericidal proteins defending the mucosal layers of the body against pathogenic microbiota. In our data, we have detected seven members of the PLUNC (palate, lung, and nasal epithelium clone) protein family. These included bactericidal/permeability-increasing proteins[51,52]. All detected BPI proteins in the saliva proteome were male biased and at least three members were characteristic of being within the top ten of the most abundant salivary proteins (Fig. 2). It is possible that various antimicrobial proteins are male biased simply to compensate for the testosterone dependent immunosuppression of reproducing males[67]. Moreover the sex-dependent resistance against bacteria (*Salmonella typhimurium*) has also been demonstrated in the house mouse where males were more resistant than females[68]. We have also detected high levels of the prolactin-inducible protein (PIP) which is a submandibular gland protein with the ability to bind immunoglobulin G (IgG), IgG-Fc, CD4-T cell receptor, and different species of bacteria (mainly streptococci), thus playing an important role in non-immune defense[69]. A natural antibiotics CRAMP was detected in individuals of both sex. CRAMP forms an amphipathic alpha-helix similar to other antimicrobial peptides, whilst functional studies showed that CRAMP is a potent antibiotics against gram-negative bacteria by inhibiting the growth of a variety of bacterial strains[61].

Kallikrein 1-related peptidases were female-biased in our data–a trend, described for the first time in wild mice. In the laboratory mouse, kallikreins seem to be male-biased and only the kallikrein 1-related peptidase b5 was female–biased[70,71]. These serine proteases are involved in the wound healing processes and have a strong antimicrobial activity[50]. Thus it is possible that the higher abundance of kallikrein 1-related peptidases in female saliva is adaptive as it may, for example, help females to maintain healthy skin development of their juveniles via kallikrein administration during a frequent allogrooming care. Moreover, we have also detected four chitinases (i.e. CH3L1, CHIA, CHIL3, CHIL4) in the saliva of males and females, which may aid removing the chitinous mouthparts of ectoprazites via administration of chitinases during the selfgrooming and allogrooming behaviour.

From the above list of proteins it is evident that saliva is a complex biological system that compromises between various functions including chemical communication, immunity and tissues repair. Because many lipocalins that we detected in the mouse saliva are known to be expressed by other tissues (e.g. nasal, lacrimal) it is likely that these proteins also act as scavengers that bind and excrete toxic compounds. We have already suggested that evolution of chemical communication and of the system of detoxification might have been driven by similar selective forces because both systems use the same pool of lipocalin transporters[27,31]. This hypothesis or as we call it the 'toxic waste hypothesis'[27] has later been suggested by another laboratory[72] with the first experimental evidence provided in a recent paper[42]. They demonstrated that mice loaded with an industrial chemical, 2,4-di-tert-butylphenol (DTBP) use MUPs for a consequent detoxification[42]. Here we suggest that the nasal and olfactory lipocalins (including MUPs) transport potential toxic waste and various degradation products from chemical signals to the oral cavity, and further to the digestive tract where they are decomposed.

To conclude, we have provided the saliva proteome from wild-living individuals of the house mouse *Mus musculus musculus*. We aimed to identify the level of sexual dimorphism in the abundance of proteins that are involved in chemical communication because most studies focused on the western house mouse subspecies (*M. m. domesticus*) and on various inbred lines. Novelty of our findings includes the detection of sexually dimorphic proteins that were previously detected only in males with MUP20 and ESP1 being a good example. For the first time, we have also shown that the saliva proteome includes proteins that are produced mainly (but not exclusively) by olfactory tissues and which are presumably transported to the oral cavity. Altogether, that makes this system (saliva) interesting and an important source of chemical signals necessary for communication as well as an interesting source of multiple markers of physiological states.

References

1. Mouse Genome Sequencing, C. *et al.* Initial sequencing and comparative analysis of the mouse genome. *Nature* **420,** 520–562, doi: 10.1038/nature01262 (2002).
2. Blanchard, A. A. *et al.* Towards further defining the proteome of mouse saliva. *Proteome science* **13,** 10, doi: 10.1186/s12953-015-0068-3 (2015).
3. Nelson, A. C., Cunningham, C. B., Ruff, J. S. & Potts, W. K. Protein pheromone expression levels predict and respond to the formation of social dominance networks. *Journal of Evolutionary Biology* **28,** 1213–1224, doi: 10.1111/jeb.12643 (2015).
4. Cunningham, C. B., Nelson, A. C., Ruff, J. S. & Potts, W. K. MUP expression is linked with sociality not competitive ability in male house mice. *Integr Comp Biol* **53,** E46–E46 (2013).
5. Novotny, M. V. Pheromones, binding proteins and receptor responses in rodents. *Biochemical Society* **31,** 117–122 (2003).
6. Timm, D. E., Baker, L. J., Mueller, H., Zidek, L. & Novotny, M. V. Structural basis of pheromone binding to mouse major urinary protein (MUP-I). *Protein Science* **10,** 997–1004 (2001).
7. Novotny, M. V., Ma, W., Wiesler, D. & Zídek, L. Positive identification of the puberty-accelerating pheromone of the house mouse: the volatile ligands associating with the major urinary protein. *Proc. R. Soc. Lond. B.* **266,** 2017–2022 (1999).
8. Shahan, K., Denaro, M., Gilmartin, M., Shi, Y. & Derman, E. Expression of six mouse major urinary protein genes in the mammary, parotid, sublingual, submaxillary, and lachrymal glands and in the liver. *Mol. Cell. Biol.* **7,** 1947–1954 (1987).
9. Stopková, R., Stopka, P., Janotová, K. & Jedelsky, P. L. Species-specific expression of major urinary proteins in the house mice (Mus musculus musculus and Mus musculus domesticus). *J Chem Ecol* **33,** 861–869 (2007).
10. Sharrow, S. D., Vaughn, J. L., Žídek, L., Novotny, M. V. & Stone, M. J. Pheromone binding by polymorphic mouse major urinary proteins. *Protein Science* **11,** 2247–2256 (2002).
11. Zidek, L. *et al.* NMR Mapping of the Recombinant Mouse Major Urinary Protein I Binding site Occupied by the Pheromone 2-*sec*-Butyl-4,5-dihydrothiazole. *Biochemistry* **38,** 9850–9861 (1999).
12. Hurst, J. L. & Beynon, R. J. Scent wars: the chemobiology of competitive signalling in mice. *BioEssays* **26,** 1288–1298 (2004).
13. Hurst, J. L. *et al.* Individual recognition in mice mediated by major urinary proteins. *Nature* **414,** 631–634 (2001).
14. Mucignat-Caretta, C. & Caretta, A. In *Advances in Chemical Communication in Vertebrates* (eds R. E. Johnston, D. Muller-Schwarze & P. Sorensen) 359–364 (Plenum Press, 1999).
15. Novotny, M. V., Harvey, S., Jemiolo, B. & Alberts, J. Synthetic pheromones that promote inter-male aggression in mice. *Proc. Natl. Acad. Sci. USA* **82,** 2059–2061 (1985).
16. Smadja, C. & Ganem, G. Subspecies recognition in the house mouse: a study of two populations from the border of a hybrid zone. *Behav. Ecol.* **13,** 312–320 (2002).
17. Smadja, C. & Ganem, G. Divergence of odorant signals within and between the two European subspecies of the house mouse. *Behavioral Ecology* **19,** 223–230 (2008).
18. Bímová, B., Albrecht, T., Macholán, M. & Piálek, J. Signalling components of mate recognition system in the house mouse. *Behavioural Processes* **80,** 20–27 (2009).
19. Mucignat-Caretta, C. *et al.* Urinary volatile molecules vary in males of the 2 European subspecies of the house mouse and their hybrids. *Chem Senses* **35,** 647–654, doi: 10.1093/chemse/bjq049 (2010).
20. Thonhauser, K. E., Raveh, S., Hettyey, A., Beissmann, H. & Penn, D. J. Scent marking increases male reproductive success in wild house mice. *Anim Behav* **86,** 1013–1021, doi: 10.1016/j.anbehav.2013.09.004 (2013).
21. Janotova, K. & Stopka, P. The level of major urinary proteins is socially regulated in wild Mus musculus musculus. *J Chem Ecol* **37,** 647–656, doi: 10.1007/s10886-011-9966-8 (2011).
22. Janotová, K. & Stopka, P. Mechanisms of chemical communication: the role of Major Urinary Proteins. *Folia Zool.* **58,** 41–55 (2009).
23. Stopka, P., Janotova, K. & Heyrovsky, D. The advertisement role of major urinary proteins in mice. *Physiology & Behavior* **91,** 667–670 (2007).
24. Rusu, A. S., Krackow, S., Jedelsky, P. L., Stopka, P. & Konig, B. A qualitative investigation of major urinary proteins in relation to the onset of aggressive behavior and dispersive motivation in male wild house mice (Mus musculus domesticus). *Journal of Ethology* **26,** 127–135 (2008).

25. Logan, D. W., Marton, T. F. & Stowers, L. Species Specificity in Major Urinary Proteins by Parallel Evolution. *PLoS ONE* **3,** doi: 10.1371/journal.pone.0003280 (2008).
26. Mudge, J. M. *et al.* Dynamic instability of the major urinary protein gene family revealed by genomic and phenotypic comparisons between C57 and 129 strain mice. *Genome Biol* **9,** R91, doi: 10.1186/gb-2008-9-5-r91 (2008).
27. Stopková, R., Hladovcová, D., Kokavec, J., Vyoral, D. & Stopka, P. Multiple roles of secretory lipocalins (MUP, OBP) in mice. *Folia Zool*. **58,** 29–40 (2009).
28. Thoß, M., Luzynski, K., Ante, M., Miller, I. & Penn, D. J. Major urinary protein (MUP) profiles show dynamic changes rather than individual 'barcode' signatures. *Frontiers in Ecology and Evolution* **3,** doi: 10.3389/fevo.2015.00071 (2015).
29. Utsumi, M. *et al.* Expression of major urinary protein genes in the nasal glands associated with general olfaction. *Journal of Neurobiology* **39,** 227–236 (1999).
30. Cavaggioni, A., Mucignat, C. & Tirindelli, R. Pheromone signalling in the mouse: role of urinary proteins and vomeronasal organ. *Archives Italiennes de Biologie* **137,** 193–200 (1999).
31. Stopkova, R. *et al.* Mouse lipocalins (MUP, OBP, LCN) are co-expressed in tissues involved in chemical communication. *Frontiers in Ecology and Evolution* **4,** doi: 10.3389/fevo.2016.00047 (2016).
32. Pes, D., Dal Monte, M., Ganni, M. & Pelosi, P. Isolation of two odorant-binding proteins from mouse nasal tissue. *Comp. Biochem. Physiol*. **103B,** 1011–1017 (1992).
33. Stopkova, R., Dudkova, B., Hajkova, P. & Stopka, P. Complementary roles of mouse lipocalins in chemical communication and immunity. *Biochem Soc T* **42,** 893–898, doi: 10.1042/Bst20140053 (2014).
34. Stopkova, R. *et al.* Novel OBP genes similar to hamster Aphrodisin in the bank vole, Myodes glareolus. *BMC Genomics* **11,** 45, doi: 10.1186/1471-2164-11-45 (2010).
35. Felicioli, A., Ganni, M., Garibotti, M. & Pelosi, P. Multiple types and forms of odorant-binding proteins in the Old-World porcupine Hystrix cristata. *Comparative biochemistry and physiology. B, Comparative biochemistry* **105,** 775–784 (1993).
36. Lazar, J., Greenwood, d. R., Rasmussen, L. E. L. & Prestwich, G. D. Molecular and Functional Characterization of an Odorant Binding Protein of the Asian Elephant, Elephas maximus: Implications for the Role of Lipocalins in Mammalian Olfaction. *Biochemistry* **41,** 11786–11794 (2002).
37. Bignetti, E. *et al.* Purification and characterisation of an odorant-binding protein from cow nasal tissue. *Eur. J. Biochem.* **149,** 227–231 (1985).
38. Spinelli, S. *et al.* The Structure of the Monomeric Porcine Odorant Binding Protein Sheds Light on the Domain Swapping Mechanism. *Biochemistry* **37,** 7913–7918 (1998).
39. Nagnan-Le Meillour, P., Vercoutter-Edouart, A. S., Hilliou, F., Le Danvic, C. & Levy, F. Proteomic Analysis of Pig (Sus scrofa) Olfactory Soluble Proteome Reveals O-Linked-N-Acetylglucosaminylation of Secreted Odorant-Binding Proteins. *Frontiers in endocrinology* **5,** 202, doi: 10.3389/fendo.2014.00202 (2014).
40. Marchese, S., Pes, D., Scaloni, A., Carbone, V. & Pelosi, P. Lipocalins of boar salivary glands binding odours and pheromones. *Eur J Biochem* **252,** 563–568 (1998).
41. Macrides, F., Clancy, A. N., Singer, A. G. & Agosta, W. C. Male hamster investigatory and copulatory responses to vaginal discharge: An attempt to impart sexual significance to an arbitrary chemosensory stimulus. *Physiology & Behavior* **33,** 627–632 (1984).
42. Kwak, J., Strasser, E., Luzynski, K., Thoss, M. & Penn, D. J. Are MUPs a Toxic Waste Disposal System? *PLoS One* **11,** e0151474, doi: 10.1371/journal.pone.0151474 (2016).
43. Cox, J. *et al.* Accurate proteome-wide label-free quantification by delayed normalization and maximal peptide ratio extraction, termed MaxLFQ. *Mol Cell Proteomics* **13,** 2513–2526, doi: 10.1074/mcp.M113.031591 (2014).
44. Mi, H., Poudel, S., Muruganujan, A., Casagrande, J. T. & Thomas, P. D. PANTHER version 10: expanded protein families and functions, and analysis tools. *Nucleic acids research* **44,** D336–D342, doi: 10.1093/nar/gkv1194 (2016).
45. Jones, D. T., Taylor, W. R. & Thornton, J. M. The rapid generation of mutation data matrices from protein sequences. *Computer applications in the biosciences: CABIOS* **8,** 275–282 (1992).
46. Tamura, K. *et al.* MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* **28,** 2731–2739, doi: 10.1093/molbev/msr121 (2011).
47. Pavelka, N. *et al.* A power law global error model for the identification of differentially expressed genes in microarray data. *BMC Bioinformatics* **5,** 203, doi: 10.1186/1471-2105-5-203 (2004).
48. Khew-Goodall, Y. *et al.* Vomeromodulin, a putative pheromone transporter: cloning, characterization, and cellular localization of a novel glycoprotein of lateral nasal gland. *FASEB J* **5,** 2976–2982 (1991).
49. Laukaitis, C. M., Dlouhy, S. R., Emes, R. D., Ponting, P. C. & Karn, R. C. Diverse spatial, temporal, and sexual expression of recently duplicated androgen-binding protein genes in Mus musculus. *BMC Evolutionary Biology* **5,** 1–16 (2005).
50. Yamasaki, K. *et al.* Kallikrein-mediated proteolysis regulates the antimicrobial effects of cathelicidins in skin. *FASEB J* **20,** 2068–2080, doi: 10.1096/fj.06-6075com (2006).
51. LeClair, E. E. Four reasons to consider a novel class of innate immune molecules in the oral epithelium. *J Dent Res* **82,** 944–950 (2003).
52. Leclair, E. E. Four BPI (bactericidal/permeability-increasing protein)-like genes expressed in the mouse nasal, oral, airway and digestive epithelia. *Biochem Soc Trans* **31,** 801–805, doi: 10.1042/ (2003).
53. Musa, M. *et al.* Differential localisation of BPIFA1 (SPLUNC1) and BPIFB1 (LPLUNC1) in the nasal and oral cavities of mice. *Cell Tissue Res* **350,** 455–464, doi: 10.1007/s00441-012-1490-9 (2012).
54. Luo, M., Fee, M. S. & Katz, L. C. Encoding pheromonal signals in the accessory olfactory bulb of behaving mice. *Science* **299,** 1196–1201, doi: 10.1126/science.1082133 (2003).
55. Berry, R. J. & Bronson, F. H. Life-History and Bioeconomy of the House Mouse. *Biological Reviews* **67,** 519–550, doi: 10.1111/j.1469-185X.1992.tb01192.x (1992).
56. Roberts, S. A. *et al.* Darcin: a male pheromone that stimulates female memory and sexual attraction to an individual male's odour. *BMC Biol*. **8,** doi: 10.1186/1741-7007-1188-1175 (2010).
57. Roberts, S. A., Davidson, A. J., McLean, L., Beynon, R. J. & Hurst, J. L. Pheromonal induction of spatial learning in mice. *Science* **338,** 1462–1465, doi: 10.1126/science.1225638 (2012).
58. Lopes, P. C. & Konig, B. Choosing a healthy mate: sexually attractive traits as reliable indicators of current disease status in house mice. *Animal Behaviour* **111,** 119–126, doi: 10.1016/j.anbehav.2015.10.011 (2016).
59. Kimoto, H. *et al.* Sex- and strain-specific expression and vomeronasal activity of mouse ESP family peptides. *Current biology: CB* **17,** 1879–1884, doi: 10.1016/j.cub.2007.09.042 (2007).
60. Kimoto, H., Haga, S., Sato, K. & Touhara, K. Sex-specific peptides from exocrine glands stimulate mouse vomeronasal sensory neurons. *Nature* **437,** 898–901 (2005).
61. Gallo, R. L. *et al.* Identification of CRAMP, a cathelin-related antimicrobial peptide expressed in the embryonic and adult mouse. *J Biol Chem* **272,** 13088–13093 (1997).
62. Laukaitis, C. M., Critser, E. S. & Karn, R. C. Salivary androgen-binding protein (ABP) mediates sexual isolation in *Mus musculus*. *Evolution* **51,** 2000–2005 (1997).
63. Jackson, B. C. *et al.* Update of the human secretoglobin (SCGB) gene superfamily and an example of 'evolutionary bloom' of androgen-binding protein genes within the mouse Scgb gene superfamily. *Hum Genomics* **5,** 691–702 (2011).

64. Porcheron, G., Garenaux, A., Proulx, J., Sabri, M. & Dozois, C. M. Iron, copper, zinc, and manganese transport and regulation in pathogenic Enterobacteria: correlations between strains, site of infection and the relative importance of the different metal transport systems for virulence. *Frontiers in cellular and infection microbiology* **3,** 90, doi: 10.3389/fcimb.2013.00090 (2013).
65. Goetz, D. H. *et al.* The Neutrophil Lipocalin NGAL is a Bacteriostatic Agent that Interferes with Siderophore-Mediated Iron Acquisition. *Molecular Cell* **10,** 1033–1043 (2002).
66. Flo, T. H. *et al.* Lipocalin 2 mediates an innate immune response to bacterial infection by sequestrating iron. *Nature* **432,** 917–921, doi: 10.1038/nature03104 (2004).
67. Klein, S. L. The effects of hormones on sex differences in infection: from genes to behavior. *Neurosci Biobehav Rev* **24,** 627–638 (2000).
68. Thonhauser, K. E., Raveh, S., Thoss, M. & Penn, D. J. Does multiple paternity influence offspring disease-resistance? *J Evol Biol*, doi: 10.1111/jeb.12854 (2016).
69. Lee, B., Bowden, G. H. W. & Myal, Y. Identification of mouse submaxillary gland protein in mouse saliva and its binding to mouse oral bacteria. *Arch Oral Biol* **47,** 327–332 (2002).
70. Lundwall, A. Old genes and new genes: the evolution of the kallikrein locus. *Thromb Haemost* **110,** 469–475, doi: 10.1160/TH12-11-0851 (2013).
71. Karn, R. C. & Laukaitis, C. M. Positive selection shaped the convergent evolution of independently expanded kallikrein subfamilies expressed in mouse and rat saliva proteomes. *PLoS One* **6,** e20979, doi: 10.1371/journal.pone.0020979 (2011).
72. Kwak, J. *et al.* Butylated hydroxytoluene is a ligand of urinary proteins derived from female mice. *Chem Senses* **36,** 443–452, doi: 10.1093/chemse/bjr015 (2011).

Acknowledgements

The research of PS team was supported by the project BIOCEV (CZ.1.05/1.1.00/02.0109) and National Program for Sustainability II (LQ1604) from the Ministry of Education, Youth and Sports. We are also grateful to Helena Uhlířová for the lab assistance, Dr. Marian Novotný for introducing us to the protein electrostatics modelling, and to Dr. Karel Harant from the Mass Spectrometry and Proteomics Service Laboratory, Faculty of Science, Charles University in Prague for performing the LC-MS/MS run.

Author Contributions

P.S. and R.S. wrote the main manuscript text, B.K. helped to prepare Figures 1–3 and with L.H. did the sample collections. P.K. produced the transcriptomic data. M.Č. had responsibilities over the animal trapping and housing. All authors have participated during experimental procedures and reviewed the manuscript.

Additional Information

Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Stopka, P. *et al.* On the saliva proteome of the Eastern European house mouse (*Mus musculus musculus*) focusing on sexual signalling and immunity. *Sci. Rep.* **6**, 32481; doi: 10.1038/srep32481 (2016).

On the tear proteome of the house mouse (*Mus musculus musculus*) in relation to chemical signalling

Romana Stopkova, Petr Klempt, Barbora Kuntova and Pavel Stopka

BIOCEV group, Department of Zoology, Faculty of Science, Charles University, Prague, Czech Republic

ABSTRACT

Mammalian tears are produced by lacrimal glands to protect eyes and may function in chemical communication and immunity. Recent studies on the house mouse chemical signalling revealed that major urinary proteins (MUPs) are not individually unique in *Mus musculus musculus*. This fact stimulated us to look for other sexually dimorphic proteins that may—in combination with MUPs—contribute to a pool of chemical signals in tears. MUPs and other lipocalins including odorant binding proteins (OBPs) have the capacity to selectively transport volatile organic compounds (VOCs) in their eight-stranded beta barrel, thus we have generated the tear proteome of the house mouse to detect a wider pool of proteins that may be involved in chemical signalling. We have detected significant male-biased (7.8%) and female-biased (7%) proteins in tears. Those proteins that showed the most elevated sexual dimorphisms were highly expressed and belong to MUP, OBP, ESP (i.e., exocrine gland-secreted peptides), and SCGB/ABP (i.e., secretoglobin) families. Thus, tears may have the potential to elicit sex-specific signals in combination by different proteins. Some tear lipocalins are not sexually dimorphic—with MUP20/darcin and OBP6 being good examples—and because all proteins may flow with tears through nasolacrimal ducts to nasal and oral cavities we suggest that their roles are wider than originally thought. Also, we have also detected several sexually dimorphic bactericidal proteins, thus further supporting an idea that males and females may have adopted alternative strategies in controlling microbiota thus yielding different VOC profiles.

Submitted 4 January 2017
Accepted 14 June 2017
Published 7 July 2017

Corresponding author
Pavel Stopka, pstopka@natur.cuni.cz

Academic editor
Monica Poelchau

Additional Information and Declarations can be found on page 16

DOI **10.7717/peerj.3541**

Subjects Bioinformatics, Evolutionary Studies, Genetics, Zoology

Keywords Pheromone, Lipocalins, Toxic waste hypothesis, Tears, Sex dimorphism, Secretoglobins, *Mus musculus musculus*, OBP, MUP, Darcin

INTRODUCTION

The genome of the mouse contains at least 55 genes for lipocalins and—due to their capacity to transport VOCs in their eight-stranded beta barrel—many of them are involved in chemical communication (*Logan, Marton & Stowers, 2008*; *Mudge et al., 2008*; *Sam et al., 2001*; *Sharrow et al., 2002*; *Stopková et al., 2009*; *Timm et al., 2001*; *Zidek et al., 1999*). MUP production is male-biased and (sub-)species-specific in the mouse urine (*Stopková et al., 2007*), and have strong effects on the reproductive success of the signaller (*Thonhauser et al., 2013*) as an honest, cheat-proof display of an individual's health and condition (*Zala et al., 2015*; *Zala, Potts & Penn, 2004*). Because they are male-biased, the signals that are transported by highly homologous and invariable MUPs (*Enk et al., 2016*; *Thoss et al., 2016*;

Thoß et al., 2015), have the capacity to regulate reproductive behaviour of female receivers (*Janotova & Stopka, 2011*; *Ma, Miao & Novotny, 1999*; *Novotny et al., 1986*; *Stopka, Janotova & Heyrovsky, 2007*), and have been proposed to function in a variety of social interactions (*Hurst & Beynon, 2004*; *Hurst et al., 2001*; *Mucignat-Caretta & Caretta, 1999*; *Nelson et al., 2015*; *Roberts et al., 2010*; *Rusu et al., 2008*). Lipocalins are also abundant in tears, e.g., MUP4, MUP5, OBP5-7, LCN11 (*Shahan et al., 1987*; *Shahan, Gilmartin & Derman, 1987*; *Stopkova et al., 2016*) and their roles in chemical communication have been particularly suggested for OBPs, namely for hamster MSP (i.e., male-specific submandibular salivary gland protein) and FLP (i.e., female lacrimal protein) which are dominantly expressed by female lacrimal glands (*Srikantan & De, 2008*; *Srikantan, Parekh & De, 2005*) and are regulated by sex hormones (*Ranganathan & De, 1995*; *Ranganathan, Jana & De, 1999*; *Srikantan, Parekh & De, 2005*). Another hamster OBP similar to MSP and FLP is Aphrodisin. It was detected in hamster vaginal secretions and presumably elicits copulatory behaviour in males (*Macrides et al., 1984*; *Singer et al., 1986*), and is also present in the urine of mole rats (*Hagemeyer et al., 2011*). MSP, FLP, and Aphrodisin are phylogenetically close to mouse OBPs (*Stopkova et al., 2014*). Mammalian OBPs were thought to be coded only by a few genes per species (*Cavaggioni & Mucignat-Caretta, 2000*; *Nagnan-Le Meillour et al., 2014*; *Pes et al., 1992*; *Pes & Pelosi, 1995*). However, there are more genes for OBPs in the mouse genome (*Stopková et al., 2009*; *Stopkova et al., 2010*) and, thus, we provided alternative names based on their position on chromosome X as *Obp1*, *Obp2*, *Obp5* (synonym in C57BL—*Obp1a* (*Pes et al., 1998*)), *Obp6*, *Obp7* (synonym in C57BL—*Obp1b* (*Pes et al., 1998*)), and *Obp8*, where *Obp3-ps* and *Obp4-ps* are pseudogenes. OBPs share typical lipocalin motif GxW as well as a specific disulfide bond (Cys38–Cys42), which represents a strong OBP-diagnostic motif CXXXC found in many mammalian OBPs including Probasin, and which are encoded by genes on the mouse chromosome X (*Srikantan, Parekh & De, 2005*; *Stopkova et al., 2014*; *Stopková et al., 2009*). Mouse *Obp* genes form two phylogenetic sub -clusters: the group-A and the group-B *Obp*s. The ancestral group-A *Obp*s include *Obp1* and *Obp2* (and *Obp3-ps*) whilst the later evolved group-B *Obp*s include *Obp5, Obp6, Obp7*, and *Obp8* (and *Obp4-ps*) (*Stopkova et al., 2016*). Furthermore, *Msp* and *Flp* are orthologs to group-A *Obps*, whilst *Aphrodisin* belongs to group-B *Obp*s (*Stopkova et al., 2010*).

In our recent study employing qPCR techniques, we have determined the mRNA expression sites for OBPs and several MUPs, and provided evidence that the exorbital lacrimal glands produce high quantities of mRNAs coding OBP5, OBP6, female-biased OBP7, and also male-biased MUP4, and non-dimorphic MUP5 and LCN11 (*Stopkova et al., 2016*). Interestingly, those lipocalins that are produced by lacrimal and nasal tissues, are finally transported to the oral cavity where they are detectable in saliva (*Stopka et al., 2016*). These proteins are also spread onto the fur with saliva during selfgrooming where they may function as chemical signals. In tears, the male-biased MUP4 is particularly important for its affinity to the male-derived pheromone 2-*sec*-butyl-4,5-dihydrothiazole—SBT (*Sharrow, Novotny & Stone, 2003*; *Sharrow et al., 2002*) which causes inter-male aggression and estrus synchrony (*Jemiolo, Harvey & Novotny, 1986*; *Novotny et al., 1985*). Male tears, however, also contain exocrine gland-secreted peptides of which ESP1 has been shown to

enhance female sexual behaviour through a specific vomeronasal receptor (*Kimoto et al., 2005*). Thus, the presence of MUP4 with its ligands and of ESP1 in the mouse tears and saliva (*Stopka et al., 2016*) may function as the signals that explain the observation of *Luo, Fee & Katz (2003)* and *Luo & Katz (2004)*, who reported that mouth and facial areas are the first and the most frequently investigated areas during mouse social contacts, which causes strong neuronal activity responses in accessory olfactory bulbs (*Luo, Fee & Katz, 2003*).

MUPs and OBPs may also be used as carriers of VOCs stemming out of metabolic and bacterial degradation, and of other potentially toxic waste in mice (*Kwak et al., 2011*; *Kwak et al., 2016*; *Larsen, Bergman & Klassonwehler, 1990*; *Petrak et al., 2007*) and in other taxa including humans (*Akerstrom et al., 2007*; *Lechner, Wojnar & Redl, 2001*), cows and pigs (*Grolli et al., 2006*), and elephants (*Lazar et al., 2002*). Thus, scavenging is seen as their parallel—and presumably ancestral—role within the 'Toxic waste hypothesis of evolution of chemical communication' (*Stopková et al., 2009*), which states that, the original function of lipocalins was to transport harmful chemicals out of the body or for their internalization in lysosomes (*Strotmann & Breer, 2011*). These compounds—and especially those that were sexually dimorphic—were an ideal source for natural selection during evolution of sexual signalling due to a link between the level of metabolic activity and individual quality.

Besides lipocalins, tears also contain antimicrobial proteins, which keep the exposed parts of the eyeball hostile to pathogens (*Walcott, 1998*; *Zoukhri, 2006*). For example, secretory IgA inhibits pathogen adhesion, phospholipase A2 hydrolyses phospholipids in bacterial membranes and various growth factors maintain cornea proliferation and regeneration, reviewed in *Fluckinger et al. (2004)*. Specific antimicrobial activity has been demonstrated for the mouse lipocalin LCN2, which is up-regulated as a response to inflammation in mucosal tissues (*Flo et al., 2004*; *Goetz et al., 2002*), and which scavenges for catecholate-type siderophores that bacteria use to sequester free iron (*Flo et al., 2004*). LCN2 is equally present in male and female saliva (*Stopka et al., 2016*). Some mechanisms of defence such as bactericidal proteins from the PLUNC (palate, lung, and nasal epithelium clone) protein family are sexually dimorphic, thus differentially defending the mucosal layers of the body against pathogenic microbiota. These include for example the bactericidal/permeability-increasing proteins - BPI (*Leclair, 2003a*; *LeClair, 2003b*) which are male-biased in the mouse saliva (*Stopka et al., 2016*). Thus, the products from defeated bacteria and from symbiotic microbiomes may be sexually dimorphic due to the sexually dimorphic expression of anti-microbial proteins. They may contribute to an existing pool of compounds that may be recognized as individual signals by which the mice recognize an individual's health (*Zala et al., 2015*; *Zala, Potts & Penn, 2004*). Chemodetection of such microorganism-associated molecular patterns (MAMPs) occurs at many places in the body including specific sets of chemosensory neurons in the mammalian nose (*Bufe & Zufall, 2016*).

The aim of this paper was to characterize the tear proteome from wild individuals of the house mouse (*M. m. musculus*). We focused on the detection of abundant and sexually-dimorphic proteins and especially on those that have the potential to transport sexual signals in their beta barrel (i.e., lipocalins) and those that may be involved in generating sex-specific VOC profiles, including e.g., antimicrobial peptides. This paper builds upon

our previous study where we identified several lipocalins across different orofacial tissues with qPCR (*Stopkova et al., 2016*), and upon our study on the saliva proteome (*Stopka et al., 2016*) where we demonstrated that many salivary proteins (e.g., LCNs, and OBPs) are not expressed by submandibular glands but are produced elsewhere in nasal and lacrimal glands/tissues. Here we further developed this aim with the state-of-the-art label-free LC-MS/MS techniques to provide further evidence on the house mouse tear protein content.

MATERIALS AND METHODS

Ethical standards

All animal procedures were carried out in strict accordance with the law of the Czech Republic paragraph 17 no. 246/1992 and the local ethics committee of the Faculty of Science, Charles University in Prague chaired by Dr. Stanislav Vybíral specifically approved this study in accordance with accreditation no. 27335/2013-17214 valid until 2019. Animals were sacrificed by cervical dislocation.

Animals

Fourteen individuals of the house mouse (the eastern form, *M. m. musculus*) used in this study were captured in the Czech Republic near Bruntál - 49.9884447N, 17.4647019E (one male, one female), in Velké Bílovice - 48.8492886N, 16.8922736E (three males, three females), Prague-Bohnice - 50.1341539N, 14.4142189E (three males, three females). All animals were trapped in human houses and garden shelters. On the day of capture or the next day, all animals were transferred to our animal facility. Each animal was caged individually with *ad libitum* access to water and food.

Tear collection

Eye lavage was used as a non-invasive method of tear collection. Both eyes were carefully rinsed with 10 μl of the saline physiology solution by a gentle pipetting and samples were then pooled. The process was repeated three times with at least a two hour interval between every rinsing, and each sample was analysed twice with MS to produce mean values from the methodology duplicates. This was done in the 'in-house' Mass Spectrometry and Proteomics Service Laboratory, Faculty of Science, Charles University in Prague.

Protein digestion

Protein samples were precipitated with the ice-cold acetone and followed by a re-suspension of dried pellets in the digestion buffer (1% SDC, 100 mM TEAB—pH $= 8.5$). Protein concentration of each lysate was determined using the BCA assay kit (Fisher Scientific, Waltham, MA, USA). Cysteines in 20 μg of proteins were reduced with a final concentration of 5 mM TCEP (60 °C for 60 min) and blocked with 10 mM MMTS (i.e., S-methyl methanethiosulfonate, 10 min Room Temperature). Samples were cleaved with trypsin (i.e., 1/50, trypsin/protein) in 37 °C overnight. Peptides were desalted on a Michrom C18 column.

nLC-MS^2 analysis

Nano Reversed phase columns were used (EASY-Spray column, 50 cm × 75 μm ID, PepMap C18, 2 μm particles, 100 Å pore size). Mobile phase buffer A was composed of

water, 2% acetonitrile and 0.1% formic acid. Mobile phase B contained 80% acetonitrile, and 0.1% formic acid. Samples were loaded onto a trap column (Acclaim PepMap300, C18, 5 μm, 300 Å Wide Pore, 300 μm × 5 mm, five Cartridges) for 4 min at 15 μl/min loading buffer was composed of water, 2% acetonitrile and 0.1% trifluoroacetic acid. After 4 min ventile was switched and Mobile phase B increased from 2% to 40% B at 60 min, 90% B at 61 min, hold for 8 min, and 2% B at 70 min, hold for 15 min until the end of run.

Eluting peptide cations were converted to gas-phase ions by electrospray ionization and analysed on a Thermo Orbitrap Fusion (Q-OT-qIT; Thermo Fisher, Waltham, MA, USA). Survey scans of peptide precursors from 400 to 1,600 m/z were performed at 120K resolution (at 200 m/z) with a 5×10^5 ion count target. Tandem MS was performed by isolation at 1.5 Th with the quadrupole, HCD fragmentation with normalized collision energy of 30, and rapid scan MS analysis in the ion trap. The MS^2 ion count target was set to 10^4 and the max injection time was 35 ms. Only those precursors with charge state 2–6 were sampled for MS^2. The dynamic exclusion duration was set to 45 swith a 10 ppm tolerance around the selected precursor and its isotopes. Monoisotopic precursor selection was turned on. The instrument was run in top speed mode with 2 s cycles.

Protein analysis

All data were analysed and quantified with MaxQuant software (version 1.5.3.8) (*Cox et al., 2014*). The false discovery rate (FDR) was set to 1% for both proteins and peptides and we specified a minimum peptide length of seven amino acids. The Andromeda search engine was used for the MS/MS spectra search against the Uniprot *Mus musculus* database (downloaded on June, 2015), containing 44,900 entries. Enzyme specificity was set as C-terminal to Arg and Lys, also allowing cleavage at proline bonds (*Rodriguez et al., 2008*) and a maximum of two missed cleavages. Dithiomethylation of cysteine was selected as fixed modification and N-terminal protein acetylation and methionine oxidation as variable modifications. The ''match between runs'' feature of MaxQuant was used to transfer identifications to other LC-MS/MS runs based on their masses and retention time (maximum deviation 0.7 min) and this was also used in all quantification experiments. Quantifications were performed with the label-free algorithms described recently (*Cox et al., 2014*) using a combination of unique and razor peptides. To detect differentially expressed / abundant proteins, we used the Power Law Global Error Model (PLGEM) (*Pavelka et al., 2004*) within the *Bioconductor package* in R software (*Gentleman et al., 2004*). This model was first developed to quantify microarray data (*Pavelka et al., 2004*); however, due to similar statistical properties—namely the distribution of signal values deviating from normality—it has proved to be an amenable model for the quantification of label-free MS-based proteomics data (*Pavelka et al., 2008*). Next, we calculated the signal-to-noise ratio—STN (equation provided in *Pavelka et al., 2008*), because it explicitly takes unequal variances into account and because it penalizes proteins that have higher variance in each class more than those proteins that have a high variance in one class and a low variance in another (*Pavelka et al., 2004*). PLGEM was fitted on a set of replicates from female data, thus setting experimental baseline. Correlation between the mean values and standard deviations was high ($r^2 = 0.96$, Pearson $= 0.94$) so we continued with the

resampled STNs and calculated differences with corresponding *p*-values between males and females.

Protein surface modelling

The surface electrostatics modelling involved several steps. First, we downloaded the structures from the RSCB Protein Data Bank (http://www.rcsb.org/) under accession IDs: 3S26, 1I04 and 2L9C, respectively. Because the mouse OBP1 structure has no record in the database we had to predict it with i-TASSER (Iterative Threading ASSEmbly Refinement) program (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) with the rat ortholog OBP1F (PDB ID: 3FIQ, 76% similarity) as template for the homologous modelling. Next, we used PyMOL - Molecular Graphic System (version 1.7.0.0) with APBS (Adaptive Poisson-Boltzman Solver) plugin to model the electrostatics with the default software settings.

RNAseq: samples, cDNA, sequencing, and analysis

Individual mice were sacrificed next day after the last tear sampling. The exorbital lacrimal glands (i.e., one gland per individual, the other one is stored) were dissected and immediately placed into RLT buffer (Qiagen, Hilden, Germany) and homogenised in MagNALyser (Roche) for 30 s at 6,000 rpm. RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufactures protocol with on-column DNase I treatment. The purity and concentration of eluted RNA was measured with a NanoDrop ND1000. The quality of RNA was checked on agarose gel electrophoresis (AGE). RNA was stored at −70 °C pending further use. For the next step, we selected only high quality samples from four male and four female replicates.

cDNA was prepared using the SMARTer PCR cDNA Synthesis Kit (Clontech, Mountain View, CA USA) and amplified with Advantage 2 PCR Kit (Clontech, Mountain View, CA USA). Both procedures were handled according to protocol for Trimmer-2 Normalization Kit (Evrogen, Moscow, Russia). The products of optimalized cDNA amplification were then loaded on AGE. For each sample, only the area of product in range from ∼400 bp to ∼1,300 bp (well visible area full of bands) was excized from the gel and the DNA products were extracted using the Gel/PCR DNA Fragments Extraction Kit (Geneaid, New Taipei City, Taiwan). Appropriate amounts of size-selected products were then secondarily amplified according to the recommended protocol from Evrogen. Products of secondary amplification were purified using MiniElute PCR Purification Kit (Qiagen, Hilden, Germany). Purified products (and the range where they emerge) were checked on AGE. Purity was analysed with NanoDrop ND1000. Concentration was measured/determined using Quant-it Pico Green dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA) and fluorimeter (Hoefer DQ 300). Rapid Library (RL) was prepared for each transcriptome (four males and four females) according to Rapid Library Preparation Manual (my454.com). Equal amounts from each of eight Rapid Libraries (10^7 molecules per μl dilution) were mixed and then used for emPCR.

We conducted 454 RNA-sequencing with a desktop pyro-sequencer GS Junior from Roche using the long reads mode. To increase the precision of transcript mapping, we excised from a gel and sequenced only transcripts between ∼400 and 1,300 bp. Transcripts of this length include those of genes, described for their involvement in chemical

communication (e.g., lipocalins). This method is amenable to further analyses because the nebulization step is skipped and, therefore, whole transcripts instead of their fragments are further pyro-sequenced and mapped. We estimated particular expression levels from the number of uniquely mapped transcripts assigned to each annotated gene. All steps followed the provider's instructions for sequencing with GS Junior (emPCR Amplification Method Manual Lib-L and Sequencing Method Manual; Roche, Basel, Switzerland). We obtained >165,000 high quality (HQ) reads. HQ 454 Reads were multiplexed, trimmed (i.e., using a trimming database that contains primers used for library preparations), filtered and aligned into contigs against *Mus musculus* cDNA database ("the super-set of all known, novel and pseudo gene predictions"; ensembl.org, 17-FEB-2015 version) and using GS Reference Mapper (Roche, Basel, Switzerland). Differential expression was analysed in R software using the *DEseq* routine within the *Bioconductor package* (*Gentleman et al., 2004*).

RNA-seq data availability

The transcriptome data is provided as bam files in 'Sequencing Read Archive' (www.ncbi.nlm.nih.gov/sra) under the accession numbers SRP063762 and BioProject PRJNA295909.

RESULTS

The tear proteome and the level of sexual dimorphism

We have generated the tear proteome of the house mouse, *M. m. musculus* and detected a total of 719 proteins at 0.01 FDR (i.e., False Discovery Rate for all peptides and proteins). First of all, we reduced our data such that only the proteins that were detected in three or more individuals were further analyzed (i.e., 457 proteins). Our aim was to identify those proteins that are sexually dimorphic (Fig. 1) and those that represent the top 5% of the most abundant proteins that may characterize the mouse tears (Fig. 2A).

PLGEM analysis of the level of sexual dimorphism revealed that 68 (14.9%) out of 457 proteins identified at 1% FDR and $p < 0.05$ were sexually dimorphic, Fig. 1. Male biased proteins included 36 (7.8%) and female biased proteins included 32 (7%) successful identifications (i.e., listed in Data S1). Thus, male-biased proteins were not more common than female-biased proteins in the tear proteome. The most significant dimorphic proteins (i.e., top 5% in Fig. 2B) included the female-biased OBP7, the male-biased MUP4, the male-only ESP1, the male-biased ESP38, and several male-biased secretoglobins (SCGB1B19, SCGB1B3, SCGB2A2—Mammaglobin, SCGB2B3, SCGB2B7). Secretoglobins are found in mammalian secretions and have important roles in the modulation of inflammation and tissue repair (*Jackson et al., 2011*), are involved in removing toxins (*Zhou et al., 2011*), and may have roles in chemical communication (*Karn & Laukaitis, 2015*). Kallikrein 1-related peptidases were also significantly sexual dimorphic (i.e., female-biased KLK1B22, KLK1B1, and KLK1B3), however, this pattern (though significant) was not consistent across all the females tested. KLKs are known as network members that are crucial for homeostasis of stratified epithelia and for activating antimicrobial Cathelicidins (*Kasparek et al., 2017*). Interestingly, we have also detected sexually dimorphic BPI proteins. Bactericidal/permeability-increasing proteins (BPI) are ~50 kDa proteins that are a part of the innate immune system, and have an antibacterial activity against the gram-negative

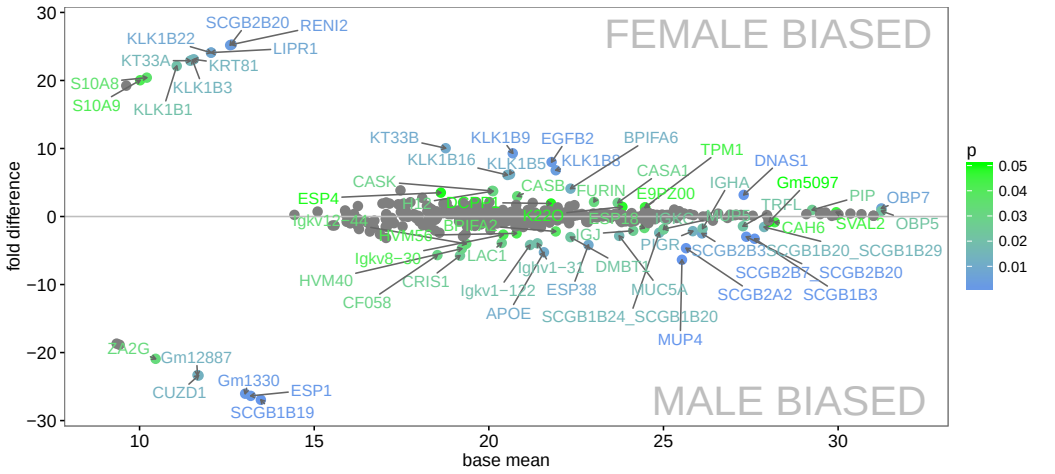


Figure 1 Graphical representation of protein signal intensities from LC-MS/MS (*X* axis) and particular fold differences between males and females. Proteins are visible as mean values of the signal (base mean peak areas) and in three clusters i.e., male-unique (below the baseline - left), female-unique (above the baseline - left), and proteins present in individuals of both sex –close to the baseline ($y = 0$). Significant differences between males and females above or below the *Y* co-ordinate (fold differences) are continuously scaled from green ($p < 0.05$) to blue ($p < 0.01$).

bacteria (*LeClair, 2003b*). We have detected three BPIs, of which BPIFA2 was male biased, BPIFA6 was female biased, whilst males and females equally expressed BPIFB9B.

The most abundant tear proteins

Based on the median value we sorted our data to detect the most abundant proteins in the tear proteome. The top 5% of the most abundant proteins that characterize the soluble tear proteome of the mouse are depicted in Fig. 2A, and include for example the female-biased lipocalins OBP5, OBP7, the unbiased lipocalins OBP1 and LCN11, and the male-biased lipocalin MUP4, Fig. 2C. Other proteins dominating the soluble tear-proteome included three male-biased secretoglobins (SCGB1B3, SCGB1B20/SCGB1B29, SCGB2B20/SCGB2B7), two unbiased secretoglobins (SCGB1B2, SCGB2B2), male-biased carbonic anhydrase 6 (CAH6), (unbiased) exocrine secreted peptide ESP6, Lacrein, and female-biased prolactin inducible protein (PIP). Interestingly, out of the top 5% most abundant proteins, ∼50% of them (i.e., 12) were significantly sexual dimorphic. Thus, even though the level of sexual dimorphism is rather low within the complete tear proteome (i.e., 15%), those few proteins that were most abundant were often the most sexually dimorphic.

Sex-unique proteins

We provide visual representation of all proteins using MA plot, also including potentially sex-unique proteins (Fig. 1), where significant points are colored from green ($p < 0.05$) to blue ($p < 0.01$). Female-unique proteins included S10A8/S10A9 which are calcium- and zinc-binding proteins and which play important roles in the regulation of inflammatory processes and immune responses, and can induce neutrophil chemotaxis and adhesion (*Vogl et al., 2007*). We have also detected the female-unique secretoglobin SCGB2B20.

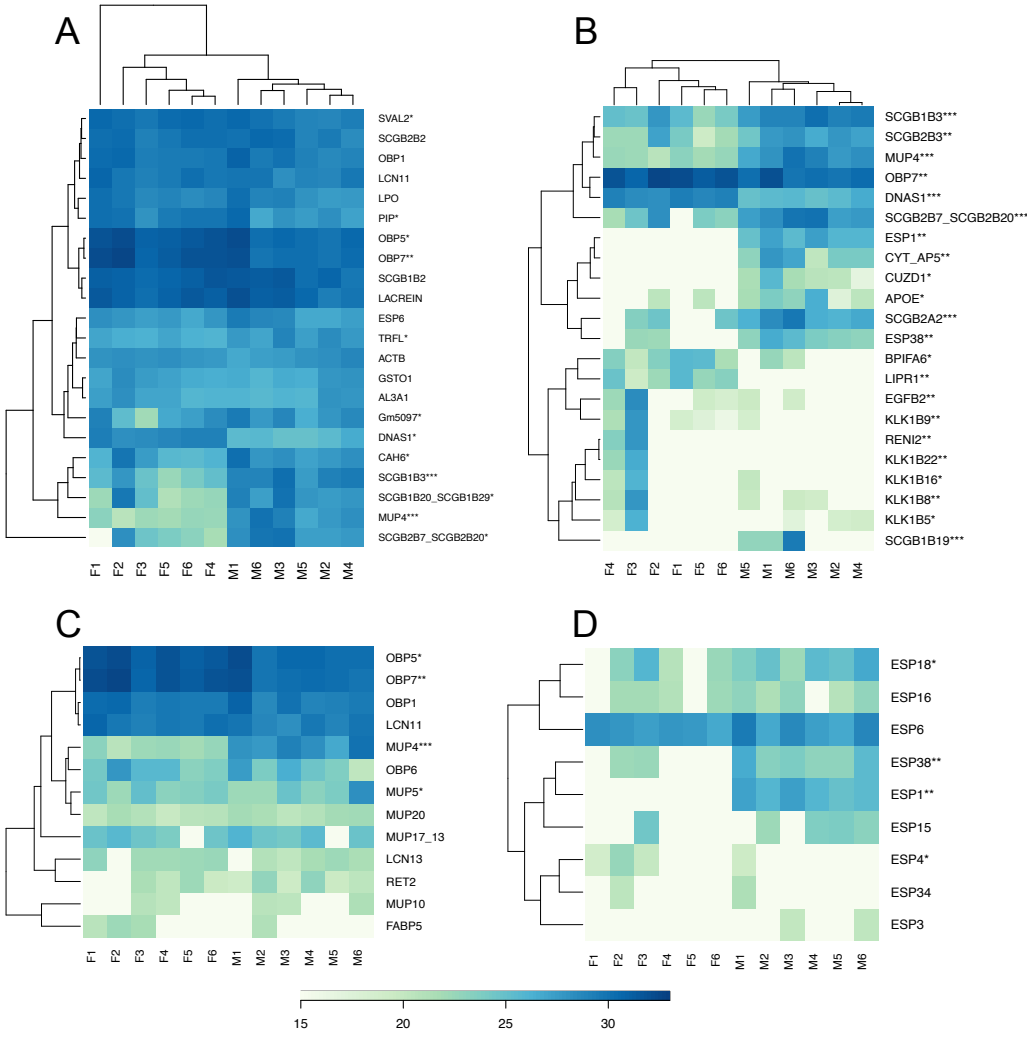


Figure 2 Graphical representations of individual variation in protein abundances with heat maps. Similarities between proteins and individuals were detected with a hierarchical clustering method: (A) the top 5% of highly expressed proteins include OBPs, SCGBs/ABPs, and ESPs; (B) the top 5% of the most significant sexually dimorphic proteins ($p < 0.02$) include ESPs, secretoglobins, lipocalins and female-biased antimicrobial protein BPIFA6. There is a notable variation between individuals in lipocalin (C) and ESP (D) abundances. Note that the expression of MUP20/Darcin is invariant over individuals. Asterisks represent: * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$).

Other female-unique proteins invlolved RENI2, LIPR1 and one keratin (KT33A). Male-unique proteins included the Secretoglobin SCGB1B19, the exocrine gland-secreted peptide ESP1, Zn-Alpha2-Glycoprotein (i.e., ZA2G), zona pellucida-like domain-containing protein 1 (CUZD1), and products of the two predicted genes Gm12887 and Gm1330. ESP1 was co-expressed with other ESPs (ESP3, ESP4, ESP6, ESP15, ESP16, ESP18, ESP34, ESP38) in tears, Fig. 2D. Though male-unique in tears, ESP1 is present in male and female saliva (*Stopka et al., 2016*). To add, various proteins that were previously detected as sex-unique now seem to be rather sex-biased and not sex-unique/limited when new

LC-MS/MS techniques with higher detection limits are employed instead of the gel-based MS techniques (e.g., *Karn & Laukaitis, 2015*).

Transcriptome: mRNAseq based analysis of exorbital lacrimal glands

To detect lipocalins that are excreted from the exorbital lacrimal glands, we performed the analysis of size-selected transcriptomes from ~400 bp to ~1,300 bp demonstrated with histograms in Fig. 3A. Top ten percent of highly expressed transcripts included *Scgb2a2*, *Pip*, *Lcrn*, *Scgb2b2*, *Scgb1b2*, *Scgb2b24*, *Spt1*, *Lcn11*, *Obp5*, Scgb2b7, *Esp6*, *Esp15*, *Scgb1b3*, *Sval2*, *Obp7*, *Scgb2b3*, *Bpifa2*, *Bglap3*, *Esp16*, *Scgb1b7*, *Gm20594 (Mtrnr2l)*, *Wfdc18* (underlined are the transcripts encoding proteins that were detected within the top 10% of highly expressed proteins). However, direct comparison between transcriptomic and proteomic datasets was not possible, because the transcriptome was prepared from selected ranges of gel extracted tissue mRNAs whilst the tear proteome over-represents the soluble fractions of all proteins. Next, we searched for sexually dimorphic genes that may account for sex-specific differences with the *DESeq* routine within the *Bioconductor package* (*Gentleman et al., 2004*). We have filtered for further analysis only the data where the sum of counts per row $\geq$10. Then, we normalised the data with a size factor vector to make the libraries comparable. Because *DESeq* calculates sexual dimorphisms from the original non-transformed number of counts we first looked at the level of variation between replicates within sex. When dispersion values are plotted against the means of the normalised counts (Fig. 3B) it is evident from the slope of the red fitting curve that data with a low mean of normalized counts have higher levels of dispersion than high expression data.

Next, we searched for differentially expressed genes by calling the *nbinomTest* in *DESeq*, vizualized in Fig. 3C. We have detected a total of 6 female-biased genes (*Obp5*, *Obp7, Obp8, Spt1, Hba*, and *Scgb2b1*) and a total of 17 male-biased genes (*Scgb2b7, Scgb1b20, Scgb1b3, Scgb1b7, Esp18, 9530002B09Rik, Scgb2b3, Esp16, Mup4, Esp24, Cyp4a12b, RP23-421B1.4, Nop10, Scgb1b28-ps, Esp1, Scgb2b20*, and *Pigr*), Fig. 3D. Next we asked which of the above sex-biased genes are most differentially expressed. Using Benjamini–Hochberg corrections we have generated new '*p*-adjusted' values. These genes (*p*-adjusted < 0.05) included a total of 13 genes with female-biased *Obp5, Obp7, Obp8*, and *Spt1*, whilst male-biased genes included *Mup4,* five secretoglobins (*Scgb2b7, Scgb1b20, Scgb1b3, Scgb1b7*, and *Scgb2b3*), two *Esp* s (*Esp16, Esp18*) and the gene *9530002B09Rik* (synonym: *Vpp1*—Ventral prostate predominant l, which was originally thought to be exclusively expressed in the prostate (*Wubah et al., 2002*)). The resulting pattern is plotted using MA plot (Fig. 3C) with red colouring of those genes that are significant at *p*-adjusted < 0.05 whilst all data with $p < 0.05$ (not corrected) are vizualized in Fig. 3D and those that have *p*-adjusted <0.05 are depicted with asterisks. Partial support for mRNA/protein concordance is provided in Fig. 3E, showing that those transcripts that are significantly sexually dimorphic and represent the soluble protein fraction are also detected on the level of protein. Furthermore, significant sexual dimorphisms of *Obp7* and *Mup4* (Figs. 3D, 3E) are in agreement with our proteomic analysis in this study and with our previously published study using qPCR (*Stopkova et al., 2016*). Our simple RNAseq analysis revealed the expression of *Obp8* which was previously detected only with bioinformatics tools (*Stopková et al., 2009*; *Stopkova et al., 2016*), thus providing the first evidence for the expression of *Obp8* transcript. Interestingly, OBP1 was

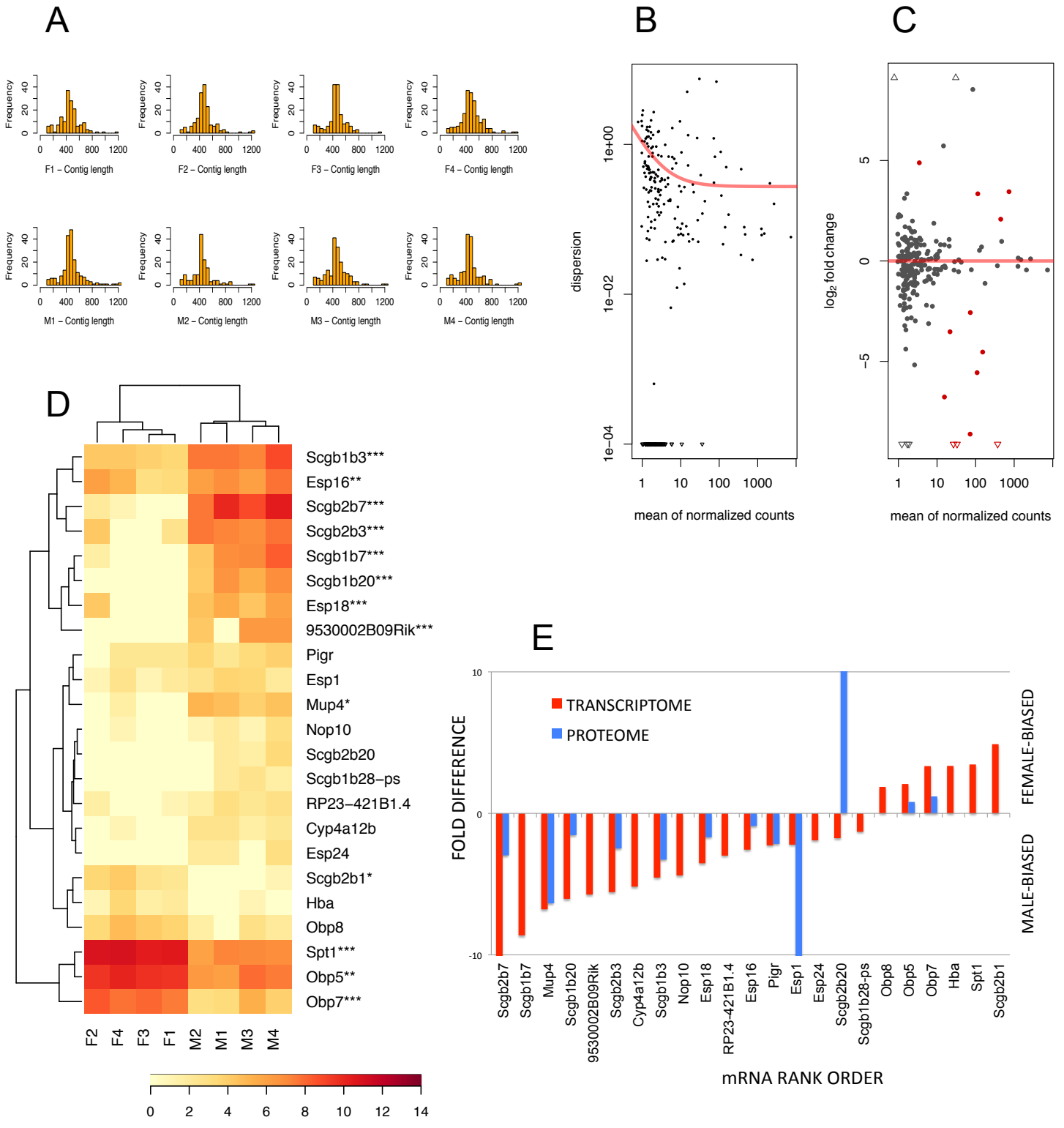


Figure 3 **The RNA-seq analysis output.** Histograms of mRNA contig lengths from size-selected transcriptomes (A) are consistent over individuals and show that more than 50% of contigs is longer than 400 bps and not exceeding 1,300 bps. (continued on next page…)

Figure 3 (…continued)
Dispersion plot (B) shows the decreasing variation in signal intensities, and along with MA plot (C) are demonstrating that the transcripts with lower number of reads have a higher dispersion. Significant sexually dimorphic abundances based on $p < 0.05$ are demonstrated with the hierarchically clustered heat map in (D), with p-adjusted values provided with asterisks ($^{*}\ P \leq 0.05$, $^{**}\ P \leq 0.01$, $^{***}\ P \leq 0.001$). (E) is demonstrating a partial support for a concordance in fold differences between mRNA expression ($p < 0.05$) and particular protein abundances for example for OBP5, OBP7, MUP4, ESPs and several secretoglobins. This relationship, however, is not linear (i.e. note huge differences in ESP1 or SCGB2B20 abundances) thus suggesting multiple sources of expression.

one of the most abundant tear proteins in individuals of both sex. However, the expression of *Obp1* transcript was medium/low in this study and low with qPCR in our previous study (*Stopkova et al., 2016*). Thus, OBP1 could be a product of several other tear-secreting glands including infra-orbital glands, accessory lacrimal glands and/or epithelial cells of ocular mucosa.

Anti-microbial peptides

BPI proteins have an antibacterial activity against gram-negative bacteria (*LeClair, 2003b*). The saliva proteome contains seven members of the bactericidal/permeability-increasing proteins (i.e., BPI *Leclair, 2003a*; *LeClair, 2003b*) which are male biased (*Stopka et al., 2016*) and include BPIA1, BPIB1, BPIB2, BPIB3, BPIFA2, BPIFB5, BPIFB9B (*Stopka et al., 2016*). However, tears only contain BPIFA2/*Bpifa2*—one of the most expressed transcripts, female-biased BPIFA6, and un-biased BPIFB9B. Thus, we searched for other proteins/peptides which may have similar roles due to their amphipathic structural properties or proteolytic activities. Recently, WFDC proteins (i.e., 'Whey acidic proteins four disulphide core') were shown to have anti-microbial properties (*Scott, Weldon & Taggart, 2011*) and the two members WFDC12 and WFDC18 are present in mouse saliva as proteins encoded by submandibular gland transcripts (i.e., *Wfdc12*, *Wfdc18*) (*Stopka et al., 2016*). In this study, we have detected WFDC12 and WFDC18 as transcripts of the exorbital lacrimal glands (i.e., *Wfdc12*, and the highly expressed *Wfdc18*), but only WFDC18 was detected in tears on the proteomic level and just in two males. Our results, however, provide evidence that the major antimicrobial protein in tears is TRFL (Lactotransferrin), Fig. 2A. Lactotransferrin also known as lactoferrin (LF) has antimicrobial properties (bactericidal, fungicidal) and is a part of the innate immune system, mainly at mucoses (*Sanchez, Calvo & Brock, 1992*). In the tear proteome, we detected the male-biased TRFL as one of the most abundant proteins and similar amounts were previously also detected in saliva (*Stopka et al., 2016*).

Homology modelling of representative lipocalin structures

OBPs and MUPs are likely to have complementary roles because OBPs are less hydrophobic and have higher iso-electric points whilst MUPs are more acidic and hydrophobic (*Stopkova et al., 2016*). In Fig. 4, we provide four representative lipocalin structures from homology modelling, thus showing that different lipocalins have similar structures but different electrostatics properties. The distribution of negative and positive residua is not random in OBP1 and even less so in LCN2. The structure of LCN2 is amphipathic because the interaction is driven by ionic strength between positively charged amino acid residues at the barrel opening of LCN2 and negatively charged siderophores. Thus, LCN2 is antimicrobial, as it efficiently scavenges for catecholate-type siderophores which bacteria

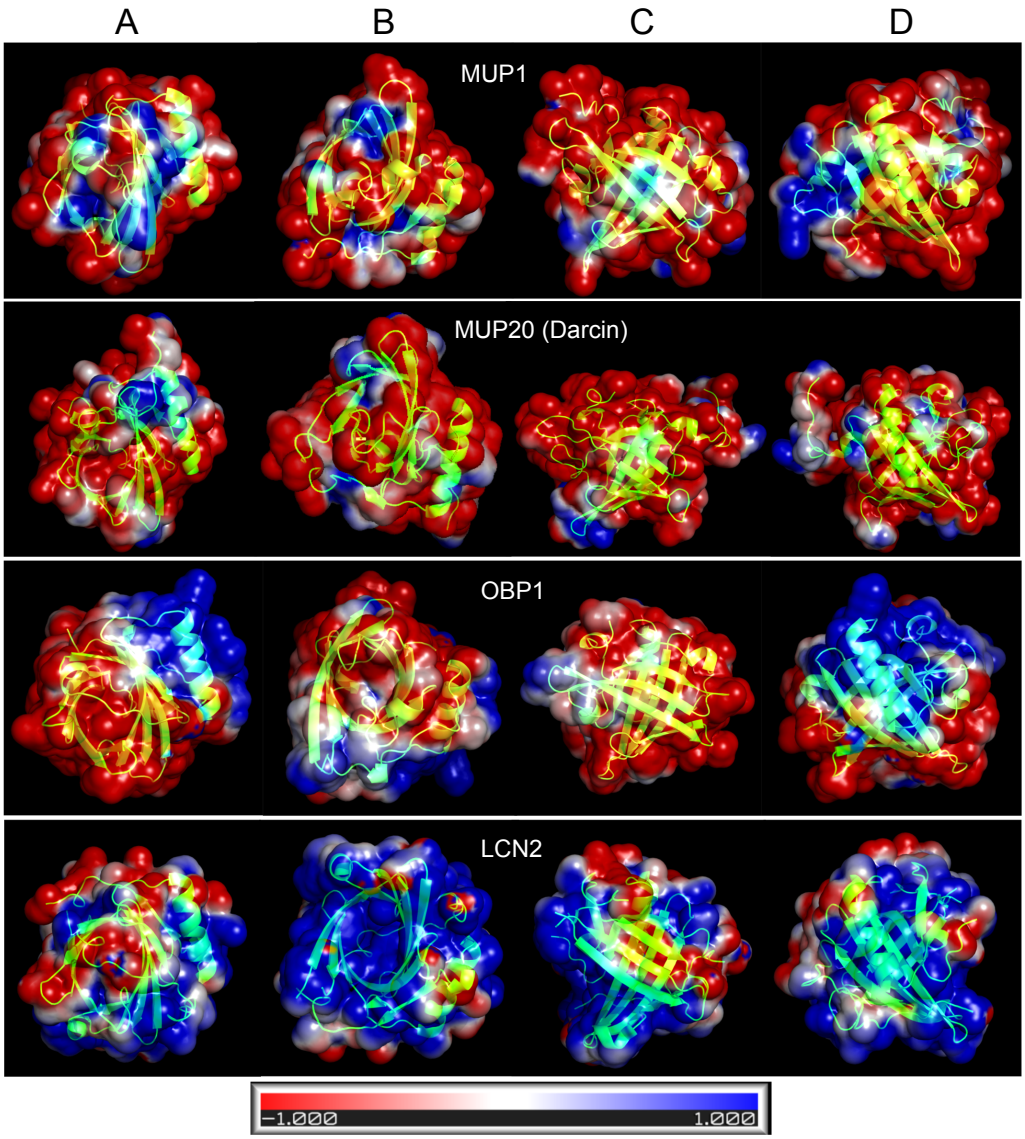


Figure 4 Graphical representation of the tertiary structure of MUP1, MUP20, OBP1, and LCN2 with electrostatics modelling, and scaled from -1kTe (red, negative) to +1kTe (blue, positive). Each protein is demonstrated in the four views: lower part of the barrel (A), opening of the barrel (B), and the two side views (C, D). Although, their structures are highly similar due to their beta-barrel structures, the distribution of positive and negative charges are non-random with OBP1 and LCN2 being amphipathic. Note the positively charged amino acid residues of LCN2 at the opening of the barrel, which bind negatively charged siderophores, whilst OBP1 has most positively charged residua on its surface and alpha helix.

produce to scavenge for free iron (*Flo et al., 2004*). The structure of OBP1, however, is amphipathic due to a non-random distribution of positively charged residua on its surface and may potentially be antimicrobial (i.e., similar to CRAMP/CAMP (*Gallo et al., 1997*)). In addition, such amphipathic structure of OBP1 may also aid to a direct attack upon negatively charged bacterial membranes by its oppositely charged OBP1 surface residua including the positively charged alpha helix.

DISCUSSION

Tears are a source of chemical signals involved in sexual signalling and are produced by sexually dimorphic lacrimal glands and their mRNAs (*Richards et al., 2006*) which code for various soluble proteins that are involved in chemical communication (*Karn & Laukaitis, 2015*; *Kimoto et al., 2005*; *Remington & Nelson, 2005*; *Sharrow, Novotny & Stone, 2003*; *Stopkova et al., 2016*). However, comparative data using label-free quantification without gel-based or Western blotting methods was to date missing. Thus, we focused on the detection of differentially abundant proteins in tears with label-free LC-MS/MS techniques to obtain more complex view on sexual signalling. We assumed that sex-specific differences in the expression of signal transporters that we detected may have roles in sexual signalling. John Maynard Smith and David Harper defined a signal as '...any act or structure which alters the behaviour of other organisms, which evolved because of that effect, and which is effective because the receiver's response has also evolved' (*Maynard Smith & Harper, 2003*). Thus, evolution of chemical communication seems to require two steps. However, the 'toxic waste hypothesis' (*Stopkova et al., 2014*; *Stopková et al., 2009*) or the theory entitled 'The origin of chemical communication by means of toxic waste perception' requires only one step because it presupposes that only the receiver's response has evolved as an adaptation to already existing sources of individual VOCs/odours which resulted from metabolic degradation. Moreover, this theory expects that the level of degradation correlates with energy intake and immune system efficiency, and thus reflects an inherent quality of the signaller.

The tear proteome of the house mouse provides a support for this hypothesis. First, the tears contain anti-microbial peptides/proteins with some of them being sexually dimorphic (e.g., BPIFA6, BPIFA2, TRFL, PIP). Thus, these proteins may yield sexually dimorphic products of bacterial degradation. Second, we have detected OBPs that are known for their capacity to scavenge for toxic substances such as 4-Hydroxynon-2-enal (HNE). HNE is a product of ocular lipid peroxidation and causes chronic inflammation (*Grolli et al., 2006*). Third, we have detected the group-A and the group-B MUPs in tears. MUPs transport pheromones and at the same time they are known to transport toxic substances out of the body (*Kwak et al., 2016*). Fourth, tear lipocalins move to the oral cavity where they were detected as proteins in the saliva where digestion starts (*Stopka et al., 2016*) including LCN3 and LCN4 which are VNO-specific (vomeronasal organ). All together, it is likely that these lipocalins may have dual functions (i.e., similarly as olfactory receptors play other roles besides the detection of chemical signals (*Ferrer et al., 2016*))—in that they are preferentially used for removing toxic substances but those that are sexually dimorphic may yield sex-specific differences which are recognized as sexual signals. Moreover, the level of sexual dimorphism in the expression of chemosensory receptors (i.e., in VNO and MOE—main olfactory epithelia) is rather limited (*Ibarra-Soria et al., 2014*). Thus, it is more likely that differential odorant detection is utilized via differential expression of chemical signal transporters.

In this study we have detected the expression of *Obp1*/OBP1, *Obp2*, *Obp6*/OBP6, and the sexually dimorphic *Obp5*/OBP5, *Obp7*/OBP7, and *Obp8* in lacrimal glands/tears. Contrary

to other *Obp*s expressed in various orofacial tissues, *Obp6* was (to date) detected only in exorbital lacrimal glands (i.e., with pyrosequencing in this study and qPCR in *Stopkova et al., 2016*). OBP5 is involved in rapid internalization of OBP-odorant complexes into lysosomes and scavenges for toxic products of free radical exposure (*Grolli et al., 2006*; *Strotmann & Breer, 2011*). However, due to the sexual dimorphism detected in this study, the presence of OBPs in tears implies their parallel roles. It is possible that all OBPs are required for the internalization of degradation products or for a transport of these harmful substances to the oral cavity where digestion starts (*Stopka et al., 2016*), whilst those that were detected as sexually dimorphic (i.e., the female-biased OBP5, OBP7, *Obp8*) may—at the same time—be essential for female sexual signalling with the products of metabolic degradation that correlate with an inherent quality of the signaller. This hypothesis, however, requires further testing.

The most interesting result of this study is evidence that males differ from females by a cocktail-like composition of significant sexually dimorphic proteins. Previously, we have demonstrated on the level of mRNA, that lacrimal glands produce high quantities of *Mup4, Lcn11, Obp5, Obp6*, and *Obp7* transcripts in the two house mouse subspecies *M. m. domesticus* and *M. m. musculus* (*Stopkova et al., 2016*). This base-line study led us to an idea that sex-specific and sex-biased expression of several different lipocalins is combinatorial, thus differentially contributing to individual scents. The combinatorial and context dependent effect of signalling has recently been described for urinary MUPs in mice (*Kaur et al., 2014*). However, in the light of new evidence, MUPs are neither polymorphic nor individually unique (*Enk et al., 2016*; *Thoss et al., 2016*; *Thoß et al., 2015*). Thus, stronger effects may be achieved by the differential expression of structurally different and sex-biased tear lipocalins with a notable variation between individuals detected in this study.

To conclude, females are characteristic of producing higher quantities of OBPs and SPT1, in tears whilst males produce more ESPs, MUPs and secretoglobins (i.e., for a comparison, see the tear and saliva proteomes of the laboratory mouse (*Blanchard et al., 2015*; *Karn & Laukaitis, 2015*)). One particular MUP - MUP20 (darcin) was surprisingly found in male and female tears and because their content is continuously moving via naso-lacrimal ducts to nasal, vomeronasal, and oral cavities where MUP20 was detected in saliva (*Stopka et al., 2016*), it is difficult to imagine that this protein functions as a protein pheromone (i.e., sensu *Roberts et al., 2010*). This is also supported by the fact that darcin is not required for sexual signalling in the laboratory mouse (*Liu et al., 2017*) and is also expressed by females in their oviductal horns and uterine liquid, thus, it is not even male-unique (*Yip et al., 2013*). Furthermore, it is possible that lipocalins (i.e., including MUP20) in the ocular tear film may have the capacity to bind air-born volatiles during social contacts and transport them to nasal tissues where they are detected as signals.

ACKNOWLEDGEMENTS

We are very grateful to Helena Uhlířová for her careful and patient lab assistance and to Karel Harant and Pavel Talacko from the Mass Spectrometry and Proteomics Service Laboratory, Faculty of Science, Charles University in Prague for performing the LC-MS/MS run.

ADDITIONAL INFORMATION AND DECLARATIONS

Funding

This research was supported by the Czech Science Foundation (GACR, P506/12/1046), and by the project BIOCEV (CZ.1.05/1.1.00/02.0109) and National Program for Sustainability II (LQ1604) from the Ministry of Education, Youth and Sports. BK was supported from a student grant SVV 260 434 / 2017. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Grant Disclosures

The following grant information was disclosed by the authors:
Czech Science Foundation: GACR, P506/12/1046.
BIOCEV: CZ.1.05/1.1.00/02.0109.
Ministry of Education, Youth and Sports: LQ1604.
SVV: 260 434 / 2017.

Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Romana Stopkova and Petr Klempt conceived and designed the experiments, performed the experiments, wrote the paper, reviewed drafts of the paper.
- Barbora Kuntova conceived and designed the experiments, performed the experiments, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.
- Pavel Stopka conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.

Animal Ethics

The following information was supplied relating to ethical approvals (i.e., approving body and any reference numbers):

All animal procedures were carried out in strict accordance with the law of the Czech Republic paragraph 17 no. 246/1992 and the local ethics committee of the Faculty of Science, Charles University in Prague chaired by Dr. Stanislav Vybíral specifically approved this study in accordance with accreditation no. 27335/2013-17214 valid until 2019. Animals were sacrificed by cervical dislocation.

DNA Deposition

The following information was supplied regarding the deposition of DNA sequences:

The transcriptome data is provided as bam files in 'Sequencing Read Archive' (www.ncbi.nlm.nih.gov/sra) under the accession numbers SRP063762 and BioProject: PRJNA295909.

Data Availability

The following information was supplied regarding data availability:

The raw data has been supplied as Data S1.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.3541#supplemental-information.

REFERENCES

Akerstrom B, Maghzal GJ, Winterbourn CC, Kettle AJ. 2007. The lipocalin alpha(1)-microglobulin has radical scavenging activity. *Journal of Biological Chemistry* **282**:31493–31503 DOI 10.1074/jbc.M702624200.

Blanchard AA, Ezzati P, Shamshurin D, Nistor AC, Leygue E, Wilkins JA, Myal Y. 2015. Towards further defining the proteome of mouse saliva. *Proteome Science* **13**:10 DOI 10.1186/s12953-015-0068-3.

Bufe B, Zufall F. 2016. The sensing of bacteria: emerging principles for the detection of signal sequences by formyl peptide receptors. *Biomolecular Concepts* **7**:205–214.

Cavaggioni A, Mucignat-Caretta C. 2000. Major urinary proteins, a_{2U}-globulins and aphrodisin. *Biochimica et Biophysica Acta* **1482**:218–228 DOI 10.1016/S0167-4838(00)00149-7.

Cox J, Hein MY, Luber CA, Paron I, Nagaraj N, Mann M. 2014. Accurate proteome-wide label-free quantification by delayed normalization and maximal peptide ratio extraction, termed MaxLFQ. *Molecular & Cellular Proteomics* **13**:2513–2526 DOI 10.1074/mcp.M113.031591.

Enk VM, Baumann C, Thoss M, Luzynski KC, Razzazi-Fazeli E, Penn DJ. 2016. Regulation of highly homologous major urinary proteins in house mice quantified with label-free proteomic methods. *Molecular BioSystems* **12**:3005–3016 DOI 10.1039/C6MB00278A.

Ferrer I, Garcia-Esparcia P, Carmona M, Carro E, Aronica E, Kovacs GG, Grison A, Gustincich S. 2016. Olfactory receptors in non-chemosensory organs: the nervous system in health and disease. *Frontiers in Aging Neuroscience* **8**:163.

Flo T, Smith KD, Sato S, Rodriguez DJ, Holmes MA, Strong RK, Akira S, Aderem A. 2004. Lipocalin 2 mediates an innate immune response to bacterial infection by sequestrating iron. *Nature* **432**:917–921 DOI 10.1038/nature03104.

Fluckinger M, Haas H, Merschak P, Glasgow B, Redl B. 2004. Human tear lipocalin exhibits antimicrobial activity by scavenging microbial siderophores. *Antimicrobial Agents and Chemotherapy* **48**:3367–3372 DOI 10.1128/AAC.48.9.3367-3372.2004.

Gallo RL, Kim KJ, Bernfield M, Kozak CA, Zanetti M, Merluzzi L, Gennaro R. 1997. Identification of CRAMP, a cathelin-related antimicrobial peptide expressed in the embryonic and adult mouse. *Journal of Biological Chemistry* **272**:13088–13093 DOI 10.1074/jbc.272.20.13088.

Gentleman Rc, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, Ellis B, Gautier L, Ge Y, Gentry J, Hornik K, Hothorn T, Huber W, Iacus S, Irizarry R, Leisch F, Li C, Maechler M, Rossini AJ, Sawitzki G, Smith C, Smyth G, Tierney L, Yang JY, Zhang J. 2004. Bioconductor: open software development for computational biology and bioinformatics. *Genome Biology* **5**:R80 DOI 10.1186/gb-2004-5-10-r80.

Goetz DH, Holmes MA, Borregaard N, Bluhm ME, Raymond KN, Strong RK. 2002. The neutrophil lipocalin NGAL is a bacteriostatic agent that interferes with siderophore-mediated iron acquisition. *Molecular Cell* **10**:1033–1043 DOI 10.1016/S1097-2765(02)00708-6.

Grolli S, Merli E, Conti V, Scaltriti E, Ramoni R. 2006. Odorant binding protein has the biochemical properties of a scavenger for 4-hydroxy-2-nonenal in mammalian nasal mucosa. *FEBS Journal* **273**:5131–5514 DOI 10.1111/j.1742-4658.2006.05510.x.

Hagemeyer P, Begall S, Janotova K, Todrank J, Heth G, Jedelsky PL, Burda H, Stopka P. 2011. Searching for major urinary proteins (MUPs) as chemosignals in urine of subterranean rodents. *Journal of Chemical Ecology* **37**:687–694 DOI 10.1007/s10886-011-9971-y.

Hurst JL, Beynon RJ. 2004. Scent wars: the chemobiology of competitive signalling in mice. *BioEssays* **26**:1288–1298 DOI 10.1002/bies.20147.

Hurst JL, Payne CE, Nevison CM, Marie AD, Humphries RE, Robertson DHL, Cavaggioni A, Beynon RJ. 2001. Individual recognition in mice mediated by major urinary proteins. *Nature* **414**:631–634 DOI 10.1038/414631a.

Ibarra-Soria X, Levitin MO, Saraiva LR, Logan DW. 2014. The Olfactory Transcriptomes of Mice. *PLOS Genetics* **10**(**9**):e1004593 DOI 10.1371/journal.pgen.1004593.

Jackson BC, Thompson DC, Wright MW, McAndrews M, Bernard A, Nebert DW, Vasiliou V. 2011. Update of the human secretoglobin (SCGB) gene superfamily and an example of 'evolutionary bloom' of androgen-binding protein genes within the mouse Scgb gene superfamily. *Human Genomics* **5**:691–702 DOI 10.1186/1479-7364-5-6-691.

Janotova K, Stopka P. 2011. The level of major urinary proteins is socially regulated in wild *Mus musculus musculus*. *Journal of Chemical Ecology* **37**:647–656 DOI 10.1007/s10886-011-9966-8.

Jemiolo B, Harvey S, Novotny M. 1986. Promotion of the Whitten effect in female mice by synthetic analogs of male urinary constituents. *Proceedings of the National Academy of Sciences of the United States of America* **83**:4576–4579 DOI 10.1073/pnas.83.12.4576.

Karn R, Laukaitis C. 2015. Comparative proteomics of mouse tears and saliva: evidence from large protein families for functional adaptation. *Proteomes* **3**:283 DOI 10.3390/proteomes3030283.

Kasparek P, Ileninova Z, Zbodakova O, Kanchev I, Benada O, Chalupsky K, Brattsand M, Beck IM, Sedlacek R. 2017. KLK5 and KLK7 Ablation Fully Rescues Lethality of Netherton Syndrome-Like Phenotype. *PLOS Genetics* **13**:e1006566 DOI 10.1371/journal.pgen.1006566.

Kaur AW, Ackels T, Kuo TH, Cichy A, Dey S, Hays C, Kateri M, Logan D, Marton T, Spehr M, Stowers L. 2014. Murine pheromone proteins constitute a context-dependent combinatorial code governing multiple social behaviors. *Cell* **157**:676–688 DOI 10.1016/j.cell.2014.02.025.

Kimoto H, Haga S, Sato K, Touhara K. 2005. Sex-specific peptides from exocrine glands stimulate mouse vomeronasal sensory neurons. *Nature* **437**:898–901 DOI 10.1038/nature04033.

Kwak J, Josue J, Faranda A, Opiekun MC, Preti G, Osada K, Yamazaki K, Beauchamp GK. 2011. Butylated hydroxytoluene is a ligand of urinary proteins derived from female mice. *Chemical Senses* **36**:443–452 DOI 10.1093/chemse/bjr015.

Kwak J, Strasser E, Luzynski K, Thoss M, Penn DJ. 2016. Are MUPs a toxic waste disposal system? *PLOS ONE* **11**:e0151474 DOI 10.1371/journal.pone.0151474.

Larsen GL, Bergman A, Klassonwehler E. 1990. A methylsulfonyl metabolite of a polychlorinated biphenyl can serve as a ligand for alpha-2-mu-globulin in rat and major-urinary-protein in mice. *Xenobiotica* **20**:1343–1352 DOI 10.3109/00498259009046632.

Lazar J, Greenwood D, Rasmussen LEL, Prestwich GD. 2002. Molecular and functional characterization of an odorant binding protein of the asian elephant, elephas maximus: implications for the role of lipocalins in mammalian olfaction. *Biochemistry* **41**:11786–11794 DOI 10.1021/bi0256734.

Lechner M, Wojnar P, Redl B. 2001. Human tear lipocalin acts as an oxidative-stress-induced scavenger of potentially harmful lipid peroxidation products in a cell culture system. *Biochemical Journal* **356**:129–135 DOI 10.1042/bj3560129.

Leclair EE. 2003a. Four BPI (bactericidal/permeability-increasing protein)-like genes expressed in the mouse nasal, oral, airway and digestive epithelia. *Biochemical Society Transactions* **31**:801–805 DOI 10.1042/bst0310801.

LeClair EE. 2003b. Four reasons to consider a novel class of innate immune molecules in the oral epithelium. *Journal of Dental Research* **82**:944–950 DOI 10.1177/154405910308201202.

Liu YJ, Guo HF, Zhang JX, Zhang YH. 2017. Quantitative inheritance of volatile pheromones and darcin and their interaction in olfactory preferences of female mice. *Scientific Reports* **7**:2094 DOI 10.1038/s41598-017-02259-1.

Logan DW, Marton TF, Stowers L. 2008. Species specificity in major urinary proteins by parallel evolution. *PLOS ONE* **3(9)**:e3280 DOI 10.1371/journal.pone.0003280.

Luo M, Fee MS, Katz LC. 2003. Encoding pheromonal signals in the accessory olfactory bulb of behaving mice. *Science* **299**:1196–1201 DOI 10.1126/science.1082133.

Luo M, Katz LC. 2004. Encoding pheromonal signals in the mammalian vomeronasal system. *Current Opinion in Neurobiology* **2004**:428–434 DOI 10.1016/j.conb.2004.07.001.

Ma W, Miao Z, Novotny MV. 1999. Induction of estrus in grouped female mice (Mus domesticus) by synthetic analogues of preputial gland constituents. *Chemical Senses* **24**:289–293 DOI 10.1093/chemse/24.3.289.

Macrides F, Clancy A, Singer A, Agosta W. 1984. Male hamster investigatory and copulatory responses to vaginal discharge: an attempt to impart sexual significance to an arbitrary chemosensory stimulus. *Physiology & Behavior* **33**:627–632 DOI 10.1016/0031-9384(84)90382-2.

Maynard Smith J, Harper DD. 2003. *Animal signals*. Oxford: Oxford University Press.

Mucignat-Caretta C, Caretta A. 1999. Protein-bound odorants as flags of male mouse presence. In: Johnston RE Muller-Schwarze, D, Sorensen P, eds. *Advances in Chemical Communication in Vertebrates*. New York: Plenum Press, 359–364.

Mudge JM, Armstrong SD, McLaren K, Beynon RJ, Hurst JL, Nicholson C, Robertson DH, Wilming LG, Harrow JL. 2008. Dynamic instability of the major urinary protein gene family revealed by genomic and phenotypic comparisons between C57 and 129 strain mice. *Genome Biology* **9**:R91 DOI 10.1186/gb-2008-9-5-r91.

Nagnan-Le Meillour P, Vercoutter-Edouart AS, Hilliou F, Le Danvic C, Levy F. 2014. Proteomic analysis of pig (Sus scrofa) olfactory soluble proteome reveals o-linked-n-acetylglucosaminylation of secreted odorant-binding proteins. *Frontiers in Endocrinology* **5**:202.

Nelson AC, Cunningham CB, Ruff JS, Potts WK. 2015. Protein pheromone expression levels predict and respond to the formation of social dominance networks. *Journal of Evolutionary Biology* **28**:1213–1224 DOI 10.1111/jeb.12643.

Novotny MV, Harvey S, Jemiolo B, Alberts J. 1985. Synthetic pheromones that promote inter-male aggression in mice. *Proceedings of the National Academy of Sciences of the United States of America* **82**:2059–2061 DOI 10.1073/pnas.82.7.2059.

Novotny MV, Jemiolo B, Harvey S, Wiesler D, Marchlewska-Koj A. 1986. Adrenal-mediated endogeneous metabolites inhibit puberty in female mice. *Science* **231**:722–725 DOI 10.1126/science.3945805.

Pavelka N, Fournier ML, Swanson SK, Pelizzola M, Ricciardi-Castagnoli P, Florens L, Washburn MP. 2008. Statistical similarities between transcriptomics and quantitative shotgun proteomics data. *Molecular & Cellular Proteomics* **7**:631–644.

Pavelka N, Pelizzola M, Vizzardelli C, Capozzoli M, Splendiani A, Granucci F, Ricciardi-Castagnoli P. 2004. A power law global error model for the identification of differentially expressed genes in microarray data. *BMC Bioinformatics* **5**:203 DOI 10.1186/1471-2105-5-203.

Pes D, Dal Monte M, Ganni M, Pelosi P. 1992. Isolation of two odorant-binding proteins from mouse nasal tissue. *Comparative Biochemistry and Physiology* **103B**:1011–1017 DOI 10.1016/0305-0491(92)90231-F.

Pes D, Mameli M, Andreini I, Krieger J, Weber M, Breer H, Pelosi P. 1998. Cloning and expression of odorant-binding proteins Ia and Ib from mouse nasal tissue. *Gene* **212**:49–55 DOI 10.1016/S0378-1119(98)00131-0.

Pes D, Pelosi P. 1995. Odorant-binding proteins of the mouse. *Comparative Biochemistry and Physiology* **112B**:471–479.

Petrak J, Myslivcova D, Halada P, Cmejla R, Cmejlova J, Vyoral D, Vulpe CD. 2007. Iron-independent specific protein expression pattern in the liver of HFE-deficient mice. *The International Journal of Biochemistry & Cell Biology* **39(5)**:1006–1015 DOI 10.1016/j.biocel.2007.01.021.

Ranganathan V, De PK. 1995. Androgens and estrogens markedly inhibit expression of a 20-Kda major protein in hamster exorbital lacrimal gland. *Biochemical and Biophysical Research Communications* **208**:412–417 DOI 10.1006/bbrc.1995.1353.

Ranganathan V, Jana NR, De PK. 1999. Hormonal effects on hamster lacrimal gland female-specific major 20 kDa secretory protein and its immunological similarity with submandibular gland major male-specific proteins. *Journal of Steroid Biochemistry and Molecular Biology* **70**:151–158 DOI 10.1016/S0960-0760(99)00103-X.

Remington SG, Nelson JD. 2005. Secretoglobins: sexually dimorphic expression of androgen-binding protein mRNA in mouse lacrimal glands. *Investigative Ophthalmology and Visual Science* **46**:31–38 DOI 10.1167/iovs.04-0216.

Richards SM, Jensen RV, Liu M, Sullivan BD, Lombardi MJ, Rowley P, Schirra F, Treister NS, Suzuki T, Steagall RJ, Yamagami H, Sullivan DA. 2006. Influence of sex on gene expression in the mouse lacrimal gland. *Experimental Eye Research* **82**:13–23 DOI 10.1016/j.exer.2005.04.014.

Roberts SA, Simpson DM, Armstrong SD, Davidson AJ, Robertson DH, McLean L, Beynon RJ, Hurst JL. 2010. Darcin: a male pheromone that stimulates female memory and sexual attraction to an individual male's odour. *BMC Biology* **8**:75 DOI 10.1186/1741-7007-8-75.

Rodriguez J, Gupta N, Smith RD, Pevzner PA. 2008. Does trypsin cut before proline? *Journal of Proteome Research* **7**:300–305 DOI 10.1021/pr0705035.

Rusu AS, Krackow S, Jedelsky PL, Stopka P, Konig B. 2008. A qualitative investigation of major urinary proteins in relation to the onset of aggressive behavior and dispersive motivation in male wild house mice (Mus musculus domesticus). *Journal of Ethology* **26**:127–135 DOI 10.1007/s10164-007-0042-3.

Sam M, Vora S, Malic B, MA W, Novotny MV, Buck LB. 2001. Odorants may arouse instinctive behaviours. *Nature* **412**:142 DOI 10.1038/35084137.

Sanchez L, Calvo M, Brock JH. 1992. Biological role of lactoferrin. *Archives of Disease in Childhood* **67**:657–661 DOI 10.1136/adc.67.5.657.

Scott A, Weldon S, Taggart CC. 2011. SLPI and elafin: multifunctional antiproteases of the WFDC family. *Biochemical Society Transactions* **39**:1437–1440 DOI 10.1042/BST0391437.

Shahan K, Denaro M, Gilmartin M, Shi Y, Derman E. 1987. Expression of six mouse major urinary protein genes in the mammary, parotid, sublingual, submaxillary, and lachrymal glands and in the liver. *Molecular and Cellular Biology* **7**:1947–1954 DOI 10.1128/MCB.7.5.1947.

Shahan K, Gilmartin M, Derman E. 1987. Nucleotide sequences of liver, lachrymal, and submaxillary gland mouse major urinary protein mRNAs: mosaic structure and construction of panels of gene-specific synthetic oligonucleotide probes. *Molecular and Cellular Biology* **7**:1938–1946 DOI 10.1128/MCB.7.5.1938.

Sharrow SD, Novotny MV, Stone MJ. 2003. Thermodynamic analysis of binding between mouse major urinary protein-I and the pheromone 2-sec-butyl-4,5-dihydrothiazole. *Biochemistry* **42**:6302–6309 DOI 10.1021/bi026423q.

Sharrow SD, Vaughn JL, Žídek L, Novotny MV, Stone MJ. 2002. Pheromone binding by polymorphic mouse major urinary proteins. *Protein Science* **11**:2247–2256.

Singer A, Macrides F, clancy AN, Agosta WC. 1986. Purification and analysis of a proteinaceous aphrodisiac pheromone from hamster vaginal discharge. *Journal of Biological Chemistry* **261**:13323–13326.

Srikantan S, De PK. 2008. Sex differences in expression and differential regulation by androgen and estrogen of two odorant-binding tear lipocalins in lacrimal glands. *General and Comparative Endocrinology* **158**:268–276 DOI 10.1016/j.ygcen.2008.07.019.

Srikantan S, Parekh V, De PK. 2005. cDNA cloning and regulation of two sex-hormone-repressed hamster tear lipocalins having homology with odorant/pheromone-binding proteins. *Biochimica et Biophysica Acta* **1729**:154–165 DOI 10.1016/j.bbaexp.2005.04.008.

Stopka P, Janotova K, Heyrovsky D. 2007. The advertisement role of major urinary proteins in mice. *Physiology & Behavior* **91**:667–670 DOI 10.1016/j.physbeh.2007.03.030.

Stopka P, Kuntova B, Klempt P, Havrdova L, Cerna M, Stopkova R. 2016. On the saliva proteome of the Eastern European house mouse (*Mus musculus musculus*) focusing on sexual signalling and immunity. *Scientific Reports* **6**:32481 DOI 10.1038/srep32481.

Stopkova R, Dudkova B, Hajkova P, Stopka P. 2014. Complementary roles of mouse lipocalins in chemical communication and immunity. *Biochemical Society Transactions* **42**:893–898 DOI 10.1042/BST20140053.

Stopková R, Hladovcová D, Kokavec J, Vyoral D, Stopka P. 2009. Multiple roles of secretory lipocalins (MUP, OBP) in mice. *Folia Zoologica* **58**:29–40 DOI 10.3409/fb58_1-2.29-34.

Stopková R, Stopka P, Janotová K, Jedelsky PL. 2007. Species-specific expression of major urinary proteins in the house mice (*Mus musculus musculus* and Mus musculus domesticus). *Journal of Chemical Ecology* **33**:861–869 DOI 10.1007/s10886-007-9262-9.

Stopkova R, Vinkler D, Kuntová B, Sedo O, Albrecht T, Suchan J, Dvorakova-Hortova K, Zdrahal Z, Stopka P. 2016. Mouse lipocalins (MUP, OBP, LCN) are co-expressed in tissues involved in chemical communication. *Frontiers in Ecology and Evolution* **4**:47 DOI 10.3389/fevo.2016.00047.

Stopkova R, Zdrahal Z, Ryba S, Sedo O, Sandera M, Stopka P. 2010. Novel OBP genes similar to hamster Aphrodisin in the bank vole, Myodes glareolus. *BMC Genomics* **11**:45 DOI 10.1186/1471-2164-11-45.

Strotmann J, Breer H. 2011. Internalization of odorant-binding proteins into the mouse olfactory epithelium. *Histochemistry and Cell Biology* **136**:357–369 DOI 10.1007/s00418-011-0850-y.

Thonhauser KE, Raveh S, Hettyey A, Beissmann H, Penn DJ. 2013. Scent marking increases male reproductive success in wild house mice. *Animal Behavior* **86**:1013–1021 DOI 10.1016/j.anbehav.2013.09.004.

Thoss M, Enk V, Yu H, Miller I, Luzynski KC, Balint B, Smith S, Razzazi-Fazeli E, Penn DJ. 2016. Diversity of major urinary proteins (MUPs) in wild house mice. *Scientific Reports* **6**:38378 DOI 10.1038/srep38378.

Thoß M, Luzynski K, Ante M, Miller I, Penn DJ. 2015. Major urinary protein (MUP) profiles show dynamic changes rather than individual 'barcode' signatures. *Frontiers in Ecology and Evolution* **3**:71 DOI 10.3389/fevo.2015.00071.

Timm DE, Baker LJ, Mueller H, Zidek L, Novotny MV. 2001. Structural basis of pheromone binding to mouse major urinary protein (MUP-I). *Protein Science* **10**:997–1004 DOI 10.1110/ps.52201.

Vogl T, Tenbrock K, Ludwig S, Leukert N, Ehrhardt C, Zoelen MA, Nacken W, Foell D, Van der Poll T, Sorg C, Roth J. 2007. Mrp8 and Mrp14 are endogenous activators of Toll-like receptor 4, promoting lethal, endotoxin-induced shock. *Nature Medicine* **13**:1042–1049 DOI 10.1038/nm1638.

Walcott B. 1998. The lacrimal gland and its veil of tears. *News in Physiological Sciences* **13**:97–103.

Wubah JA, Fischer CM, Rolfzen LN, Khalili M, Kang J, Green JE, Bieberich CJ. 2002. Ventral prostate predominant I, a novel mouse gene expressed exclusively in the prostate. *Prostate* **51**:21–29 DOI 10.1002/pros.10060.

Yip KS, Suvorov A, Connerney J, Lodato NJ, Waxman DJ. 2013. Changes in mouse uterine transcriptome in estrus and proestrus. *Biology of Reproduction* **89**(**1**):13 DOI 10.1095/biolreprod.112.107334.

Zala SM, Bilak A, Perkins M, Potts WK, Penn DJ. 2015. Female house mice initially shun infected males, but do not avoid mating with them. *Behavioral Ecology and Sociobiology* **69**:715–722 DOI 10.1007/s00265-015-1884-2.

Zala SM, Potts WK, Penn DJ. 2004. Scent-marking displays provide honest signals of health and infection. *Behavioral Ecology* **15**:338–344 DOI 10.1093/beheco/arh022.

Zhou X, Wei YA, Xie F, Laukaitis CM, Karn RC, Kluetzman K, Gu J, Zhang QY, Roberts DW, Ding XX. 2011. A novel defensive mechanism against acetaminophen toxicity in the mouse lateral nasal gland: role of CYP2A5-mediated regulation of testosterone homeostasis and salivary androgen-binding protein expression. *Molecular Pharmacology* **79**:710–723 DOI 10.1124/mol.110.070045.

Zidek L, Stone MJ, Lato SM, Pagel MD, Miao Z, Ellington A, Novotny M. 1999. NMR Mapping of the Recombinant Mouse Major Urinary Protein I Binding site Occupied by the Pheromone 2-*sec*-Butyl-4,5-dihydrothiazole. *Biochemistry* **38**:9850–9861 DOI 10.1021/bi990497t.

Zoukhri D. 2006. Effect of inflammation on lacrimal gland function. *Experimental Eye Research* **82**:885–898 DOI 10.1016/j.exer.2005.10.018.

The olfactory transcriptome and proteome of the house mouse (*Mus musculus musculus*)

Barbora Kuntová[1], Romana Stopková[1] & Pavel Stopka[1,*]

[1] *BIOCEV group, Department of Zoology, Faculty of Science, Charles University, Viničná 7, CZ 12844, Czech Republic*
[*] Correspondence: Pavel Stopka pstopka@natur.cuni.cz

Abstract
Mammalian olfaction depends on chemosensory neurons of the main olfactory epithelia (MOE), and/or of the accessory olfactory epithelia in the vomeronasal organ (VNO). Thus, we have generated the VNO and MOE transcriptomes and the soluble proteome of nasal cavity. Due to a low sexual dimorphism in MOE and VNO transcripts, the sex-specific sensing seems less likely. However, olfaction may depend on additional mechanisms that are involved in removing the background compounds from the sites of detection. Odorant binding proteins (OBPs) are thought to be involved in this process and in our study they belonged to the most expressed transcripts along other lipocalin genes (e.g. *Lcn13, Lcn14*) and antimicrobial proteins. OBPs were highly abundant with only few being sexually dimorphic. We have also detected the male-biased major urinary proteins MUP4 and MUP5, group-B MUPs that were thought to be abundant only in the urine, and the exocrine gland-secreted peptides ESP1 and ESP22 that were male-biased but not sex-unique in the nose. For the first time, we demonstrate, that the expression of lipocalins correlates with particular antimicrobial proteins thus suggesting that their individual variation is driven by natural microbiota and by pathogens that regularly enter the body. Moreover, along chemosignalling, lipocalins, including OBPs and MUPs, are likely to be involved in the antimicrobial defence as detoxifying devices along the 'eyes-nose-oral cavity' axis.

Key words: VNO, MOE, olfactory, mouse, lipocalin, chemical communication, immunity, sensory

Introduction

Chemical communication of the house mouse is mediated by the production of sex-biased major urinary proteins (MUP) from the lipocalin family, that due to their beta-barrel structure are able to protect and transport volatile pheromones in their hydrophobic pockets (Zidek et al., 1999;Timm et al., 2001;Sharrow et al., 2002;Sharrow et al., 2003). MUPs, are deposited with urine marks (Jemiolo et al., 1992), and their ligands are detectable with chemosensory neuronal receptors in MOE and VNO (Buck and Axel, 1991;Moss et al., 1997;Buck, 2000;Leinders-Zufall et al., 2000). These receptors are differentially excitable under different pH (Cichy et al., 2015). The signal containing secretions such as urine and saliva yield strain specific responses at the accessory olfactory bulb (Kahan and Ben-Shaul, 2016) and female-estrus-state specific responses to both saliva and vaginal secretions (Ben-Shaul et al., 2010). These responses yield differential sensory representations in the medial amygdala (Bergan et al., 2014), and are responsible for physiological and behavioural effects in the receiver such as estrus induction and synchronization described

elsewhere (Jemiolo et al., 1986;Jemiolo et al., 1989;Jemiolo and Novotny, 1994;Ma et al., 1999;Novotny et al., 1999a;Novotny et al., 1999b;Sam et al., 2001). The genes for chemosensory neuronal receptors are encoded by ~1700 genes and pseudogenes in the mouse genome (Ibarra-Soria et al., 2014). It has been shown that the olfactory transcriptomes are only minimally different between males and females (Ibarra-Soria et al., 2014). However, other genes with inter-individual variation – mainly from the lipocalin family – expressed in nasal tissues (Shiao et al., 2012;Ibarra-Soria et al., 2014;Stopkova et al., 2016) may also have roles in olfaction and, thus, we aimed to determine the expression pattern of these detected genes in wild derived mice, *Mus musculus musculus*, and to provide sufficient evidence for their expression on the level of soluble proteome of the mouse nasal mucosa.

Genes for MUPs are organized in a cluster on the chromosome 4 (Logan et al., 2008;Mudge et al., 2008), most of them are highly homologous in *M. m. musculus* (Thoß et al., 2015;Enk et al., 2016;Thoss et al., 2016), and have higher expression levels upon social contacts or in social groups (Stopka et al., 2007;Janotova and Stopka, 2011;Enk et al., 2016). They are sexually dimorphic in the house mouse (Knopf et al., 1983;Sampsell and Held, 1985) and the level of sex-dimorphism is sub-species specific (Stopková et al., 2007;Hurst et al., 2017). MUPs are also known to vary throughout the estrous cycle in the urine of females (Janotova and Stopka, 2011), and thus are important components of sexual signalling. MUPs and other lipocalins (e.g. odorant binding proteins, OBPs) are also present in the orofacial areas of the mouse head, namely in tears (Stopkova et al., 2017) and saliva (Stopka et al., 2016) as the products of lacrimal, nasal, salivary and various lymphoid and mucosal glands. Particularly, we have determined the expression pattern of several MUPs and OBPs in orofacial tissues and provided evidence that lacrimal glands produce high quantities of *Mup4, Lcn11, Obp5, Obp6,* and *Obp7* transcripts in the two house mouse subspecies *M. m. domesticus* and *M. m. musculus* (Stopkova et al., 2016).

On the level of proteins, females are characteristic of producing higher quantities of OBPs in tears whilst males produce more exocrine gland-secreted peptides - ESPs, MUPs and secretoglobins – SCGBs (Stopkova et al., 2017). MUPs and OBPs are also detected in saliva, though OBPs are not coded by genes in submandibular glands (Stopka et al., 2016), and thus it is likely that MUPs and OBPs are involved in the transport of particular ligands along the 'eyes-nose-oral cavity' axis. Saliva, thus represent a complex mixture of proteins with their ligands where they may function as a cocktail-like combinatorial source of individual chemical signals that are detected directly by the receiver or may be spread on the fur during selfgrooming, where their ligands may also act as signals (Stopka et al., 2016). Thus, one of the aims of this study was to further investigate whether lipocalins are also present in the nose and whether they are coded by genes expressed in MOE and/or VNO, or whether other tissues produce them (e.g. lacrimal glands) and then they are transported to nasal mucosa where they are detected with chemosensory receptors.

A primary site of signal detection in mammals is the nostrils. Similarly, pathogens enter animal bodies the same way. Therefore similar evolutionary forces might have shaped the evolution of systems for recognition of pathogens and chemical signals (Stopkova et al., 2014). Ligands associated with bacterial infections and those that are products of defeated bacteria during regulation of microbiota are also sensed via MOE and VNO via their microorganism-associated molecular patterns (MAMPs),

and they are sensed in many places in the body including specific sets of chemosensory neurons in the mammalian nose (Bufe and Zufall, 2016). They also include the formyl peptide receptor-like proteins in VNO, which provide sensitivity to disease/inflammation-related ligands (Riviere et al., 2009) and presumably are responsible for the activation of bactericidal proteins. Bactericidal proteins (i.e. such as BPI proteins) were previously detected in the olfactory transcriptomes of the mouse (Ibarra-Soria et al., 2014) in tears (Stopkova et al., 2017) and saliva (Stopka et al., 2016) and thus, our aim was to detect a wider network of antimicrobial proteins in nasal tissues and to determine to which extent their expression may explain the variation in the expression of lipocalins of the nasal cavity.

Materials and methods

Ethical Standards

All animal procedures were carried out in strict accordance with the law of the Czech Republic paragraph 17 no. 246/1992 and the local ethics committee of the Faculty of Science, Charles University in Prague chaired by Dr. Stanislav Vybíral specifically approved this study in accordance with accreditation no. 27335/2013-17214 valid until 2019.

Subjects, housing conditions and sample collection

In this experiment, we used a total of 10 G1 wild-derived *Mus musculus musculus* males and females (90 days old) with food provided ad libitum and under stable condition (i.e. 13:11 hrs, D:N, temperature t=23°C). Protein samples were collected via nasal lavage with gentle pipetting by flushing in and out of the nose 10ul of distilled water during three-second intervals with 10ul (white) pipette tips. This procedure was repeated three times per mouse.

The transcriptome

We used 12 individual house mice (different but of the same age and weight to those used for the protein collection). The vomeronasal organ and olfactory epithelia (i.e. mixed samples from left and right sides) were dissected and immediately placed into RLT buffer (Qiagen) and homogenised in MagNALyser (Roche) for 30s at 6000rpm. RNA was isolated using the RNeasy Mini Kit (Qiagen) according to the manufactures protocol with on-column DNase I treatment. The purity and concentration of eluted RNA was measured with NanoDrop ND1000. The quality of RNA was checked with High Sensitivity DNA Assay on 2100 Bioanalyzer (Agilent). RNA was stored at -70°C pending further use. For the next step, we selected only high quality samples (RIN ~ 8) from six male and five female individual replicates each containing the two tissues (MOE, VNO). cDNA library was prepared with TruSeq Stranded mRNA LT Sample Prep Kit (i.e. a total of 22 samples / two kits). Illumina MiSeq sequencing was performed with MiSeq® Reagent Kit v3 (600 cycle).

Data organization and manipulation

Illumina MiSeq fastq files (stored in BaseSpace server) were used for filtering and trimming the paired end reads with Cutadapt, which finds and removes adapter sequences, primers, poly-A tails and other types of unwanted sequence from sequencing reads. We set the minimum read length to 30bp, trimming quality threshold was set to 30, and 10 nucleotides were removed from the 5' ends. Second process of the computational part was mapping of these reads to the reference genome. We used STAR for mapping individual sequences to the reference genome

of *Mus musculus* (GRC38). Maximum number of mismatches threshold was set to 5.0 whilst 0.5 was used as the lowest level for the normalization of alignment score to a read length and for the normalization of numbers of matched bases to read length. The genome mapping generated output files (*.sam), which we converted to bam files, and sorted them using SAMtools. The number of fragments aligned to each gene was counted using the HTSeq package with the script *htseq-count*. HTSeq was thus used to generate the input files (i.e Count tables) for further analyses. These tables contain Ensembl gene id as well as the gene names, and are provided in Supplementary Dataset.

Differential expression analysis

Differential expression was analysed in R software using the *DEseq* routine within the *Bioconductor package* (Gentleman et al., 2004). Variation between replicates was calculated with the function *estimateDispersions*, using *per-condition* as the method. Genes were considered to be differentially expressed if they had an adjusted p-value of 0.05 or less (equivalent to a false discovery rate <5%). The data was normalised with a size factor vector to make the libraries comparable. Because *DESeq* calculates sexual dimorphisms from the original non-transformed number of counts we first looked at the level of variation between replicates within sex. When dispersion values are plotted against the means of the normalised counts it is common that data with a low mean of normalized counts have higher levels of dispersion than high expression data. We used the expectation-maximization algorithm provided in the *Mixtools* Bioconductor package, using all genes with at least one fragment count in one replicate, for each of the two tissues. Thus, we used *Mixtools* to identify a mixture of normal distributions within the negatively binomial distribution of our data.

RNA-seq data availability

The transcriptome data is provided as bam files in 'Sequencing Read Archive' (www.ncbi.nlm.nih.gov/sra) under the accession number – XXXXXX, BioProject: XXXXX.

Protein Digestion

All protein samples were cold-acetone precipitated and centrifuged at 10 000 rcf for 10minutes, 0°C. This was followed by a re-suspension of dried pellets in the digestion buffer (1% SDC, 100mM TEAB – pH=8.5). Protein concentration of each lysate was determined using the BCA assay kit (Fisher Scientific). Cysteines in 20µg of proteins were reduced with a final concentration of 5mM TCEP (60° C for 60 min) and blocked with10mM MMTS (i.e. S-methyl methanethiosulfonate, 10 min Room Temperature). Samples were cleaved with trypsin (1 ug of trypsin per sample) in 37°C overnight. Peptides were desalted on a Michrom C18 column.

nLC-MS^2 Analysis

Nano Reversed phase columns were used (EASY-Spray column, 50 cm x 75 µm ID, PepMap C18, 2 µm particles, 100 Å pore size**)**. Mobile phase buffer A was composed of water, and 0.1% formic acid. Mobile phase B contained acetonitrile, and 0.1% formic acid. Samples were loaded onto a trap column (Acclaim PepMap300, C18, 5 µm, 300 Å Wide Pore, 300 µm x 5 mm, 5 Cartridges) for 4 min at 15 µl/min loading buffer was composed of water, 2% acetonitrile and 0.1% trifluoroacetic acid. After 4 minutes ventile was switched and Mobile phase B increased from 4% to 35% B at 60

min, 75% B at 61 min, hold for 8 minutes, and 4% B at 70 min, hold for 15 minutes until the end of run.

Eluting peptide cations were converted to gas-phase ions by electrospray ionization and analysed on a Thermo Orbitrap Fusion (Q-OT-qIT, Thermo). Survey scans of peptide precursors from 350 to 1450 *m*/*z* were performed at 120K resolution (at 200*m*/*z*) with a 5×10^5 ion count target. Tandem MS was performed by isolation at 1.5 Th with the quadrupole, HCD fragmentation with normalized collision energy of 30 and rapid scan MS analysis in the ion trap. The MS^2 ion count target was set to 10^4 and the max injection time was 35ms. Only those precursors with charge state 2–6 were sampled for MS^2. The dynamic exclusion duration was set to 45s with a 10ppm tolerance around the selected precursor and its isotopes. Monoisotopic precursor selection was turned on. The instrument was run in top speed mode with 2s cycles.

Protein analysis

LC-MS data were analysed and quantified with MaxQuant software (version 1.5.3.8) (Cox et al., 2014). The false discovery rate (FDR) was set to 1% for both proteins and peptides and we specified a minimum peptide length of seven amino acids. The Andromeda search engine was used for the MS/MS spectra search against our modified Uniprot *Mus musculus* database (downloaded on June, 2015), containing 44,900 entries. We modified our databases such that all MUP, OBP sequences were removed and instead of them we have added a complete list of MUPs from Ensembl database, and OBPs from NCBI (sensu - citation (Stopkova et al., 2016)). Next we added some Tremble sequences that were missing in Uniprot, for example KLKs, BPIs, SPINKs, SCGB/ABPs, and LCNs. Enzyme specificity was set as C-terminal to Arg and Lys, also allowing cleavage at proline bonds (Rodriguez et al., 2008) and a maximum of two missed cleavages. Dithiomethylation of cysteine was selected as fixed modification and N-terminal protein acetylation and methionine oxidation as variable modifications. The "match between runs" feature of MaxQuant was used to transfer identifications to other LC-MS/MS runs based on their masses and retention time (maximum deviation 0.7 min) and this was also used in all quantification experiments. Quantifications were performed with the label-free algorithms described recently (Cox et al., 2014) using a combination of unique and razor peptides. All statistical analyses were performed in R software (Crawley, 2007). First, the dataset was normalized to diminish potential differences due to differential protein extractability and also due to potential differences caused by different signal intensity between samples. We used a normalization based upon quantiles, which normalizes a matrix of peak areas / intensities with the function normalize.quantiles from 'preprocessCore' routines under the Bioconductor package (Bolstad et al., 2003). This method is based upon the concept of a quantile-quantile plot extended to n dimensions. To check that the data distribution conforms to the same type of distribution after normalization, we used 'mixtools' (Gentleman et al., 2004). Second, we used the Power Law Global Error Model (PLGEM) (Pavelka et al., 2004) to detect differentially expressed / abundant proteins using the functions plgem.fit and plgem-stn (Gentleman et al., 2004). This model was first developed to quantify microarray data (Pavelka et al., 2004), however, due to similar statistical properties – namely the n-binomial distributions of signal values (i.e. deviating from normality) – it has proved to be an amenable model for the quantification of label-free MS-based proteomics data (Pavelka et al., 2008). We calculated the signal-to-noise ratio – STN (equation provided in citation (Pavelka et al., 2008)), because it explicitly takes

unequal variances into account and because it penalizes proteins that have higher variance in each class more than those proteins that have a high variance in one class and a low variance in another (Pavelka et al., 2004). We continued with the resampled STNs and calculated differences with corresponding p-values between males and females. Original and normalized LC-MS/MS data are provided in Supplementary Dataset. For our multiple correlation analysis we used Pearson correlations and the Benjamini-Hochberg p-adjusted values using the '*psych*' routines under the Bioconductor package (Bolstad et al., 2003).

Results

Transcriptome: mRNA-seq based analysis of MOE and VNO

Similarly as in citation (Ibarra-Soria et al., 2014), we have detected a mixture of two normal distributions (i.e. low-expression (red model-fitting curve) and high-expression (green curve) data, Figure1A,B) and obtained the posterior p-values with which particular data points fall onto one or another distribution within the mixture of the two normal distributions. For further analyses, we have reduced our datasets such that those points that on the level of $p<0.05$ have fallen to a low-expression data distribution (i.e. the red fitting curve in Figure1A,B) were not analysed. The level of sexual dimorphism in the expression of the MOE and VNO transcripts was extremely low. Only 7 out of a total of 12023 transcripts (0.06%) were sexually dimorphic in MOE (the 4 male-unique transcripts included *Eif2s3y, Kdm5d, Ddx3y, Uty*, and the male-biased *Pon1*, and the female-unique transcript *Xist* and the female-biased transcript *Cox8b*). A total of 13 out of 13510 expressed transcripts (0.1%) were sexually dimorphic in VNO with the male-uniqe *Eif2s3y, Ddx3y, Kdm5d, Uty,* and male-biased *Stmn4,* and with female-unique *Xist,* and female-biased *Lum, Fn1, Mfsd4a, Aebp1, Mmp2, Aqp1,* and *Col12a1*. Underlined are the transcripts that were detected as sex-unique in both datasets (i.e. MOE and VNO). They are coded by genes on sex chromosomes, which is in agreement with a previous study (Ibarra-Soria et al., 2014), however no lipocalins were detected as sexually dimorphic in this study, though some of them revealed a trend on $p<0.05$ (e.g. female-biased *Obp7, Mup5*), but when compared to other genes and using the p-adjusted values they are no longer significant.

The distribution of highly expressed genes was different in MOE and VNO. In VNO the first 50 genes (i.e. from 13510 genes) accounted for 50% of all fragments whilst in MOE the distribution is less extreme with 250 genes (i.e. from 12023 genes) accounting for 50% fragments in the original dataset. Lipocalins accounted for 34% of all fragments in VNO including the most abundant genes (i.e. in decreasing order) – *Lcn14, Lcn13, Lcn3, Lcn4, Obp1, Obp2, Obp5, Mup4* etc. In MOE lipocalins accounted for 16% of all fragments with most abundant genes being – *Obp2, Obp1, Obp5, Obp8, Mup4, Lcn13, Lcn14, Lcn11, Mup5, Obp7, Obp3-p, Lcn3* etc. Antimicrobial proteins also represented highly expressed genes. VNO is characterized by *Wfdc18, Bpifa1, Bpifb9a, Bpifb9b* (1% of all fragments) whilst in MOE, we have detected highly expressed *Bpifb9a*, *Bpifb9b, Bpifa1, Bpifb3, Wfdc18, Bpifb5, Bpifb4, Bpifb6, Bpifb1* (11% of all fragments).

Our sequencing depth with Illumina MiSeq did not allow for a detailed study of receptors. However, even with our data we can confirm that wild-derived house mice express the olfactory receptor *Olfr124* in VNO more than in MOE. Furthermore, higher repertoire of formyl-peptide receptors was detected in VNO (*Fpr-rs3*, *Fpr-rs6,*

Fpr-rs7, Fpr2, and *Fpr1*) whilst in MOE we have detected just *Fpr1* and *Fpr2* and six *Taar* receptor genes (i.e. none was detected in VNO). More information is provided in raw data of the Supplementary dataset.

Soluble proteome of the nasal cavity

We have generated the proteome of the nasal cavity of the house mouse, *M. m. musculus* and detected a total of 673 proteins at 0.01 FDR (i.e. False Discovery Rate for all peptides and proteins). First of all, we reduced our data such that only the proteins that were detected in three or more individuals and with median expression per row being higher than 1 were further analyzed (i.e. 517 proteins). Our aim was to identify those proteins that are sexually dimorphic (Figure 2) and those that represent the top 5% of the most abundant proteins that may characterize the proteome of the mouse nasal cavity. To reduce the influence of the false positive abundances due to differences in initial signal intensities between individuals, we quantile-normalized a matrix of protein abundances with 'preprocessCore' routine within the *Bioconductor package* in R software (Gentleman et al., 2004); this step ensures that differential expression (i.e. abundance) is measured instead of differential extractability of proteins from nasal mucosal secretions. This normalization strategy has resulted in highly similar datasets with similar data distribution (Figure 2A) thus decreasing the potential of obtaining false positive values.

Next, we searched for differentially expressed proteins between males and females using the Power Law Global Error Model - PLGEM (Pavelka et al., 2004). Mean value differences are visualized with MA plot in Figure 2B and in the volcano plot in Figure 2C (only protein names with $P<0.05$ and fold change $FC>2$ are shown). Complete lists of proteins and corresponding p-values are provided in the Supplementary Dataset. The most surprising result of this study is the finding that the level of sexual dimorphism was much higher on the level of proteins than on the level of transcripts. A total of 87 out of 517 proteins (16.8%) were sexually dimorphic with 45 proteins (8.7%) being male biased and 42 proteins (8.1%) being female biased. This is similar to the level of sexual dimorphism that we recently detected in the house mouse tears (Stopkova et al., 2017). When a proportional measure is used, lipocalins accounted for a total of 36.8% of all male proteins, whilst in females, lipocalins accounted for a total of 46.4% of all proteins. OBPs are proportionally more common than MUPs (OBPs: males 33.6%, females 42.3%; MUPs: males 0.91%, females 0.43). When looking at antimicrobial proteins, BPIFB9B accounted for 19.2% of all proteins in males and 12.3% in females. When all antimicrobial proteins are counted, a total of 24.1% was detected in males and 16.6% in females. Thus lipocalins and antimicrobial proteins accounted for the majority of proteins detected in nasal secretions of the mouse (i.e. >50%).

Gene/protein ontology of sexually dimorphic proteins

A total of 42 proteins was significantly female biased in the nasal cavity proteome. The String database revealed significant interactions between a total of 37 proteins that were female biased (PPI enrichment p-value: 0.000275). Some of those proteins (12 RS3A, RL12, PLEC, EVPL, PEPL, SCEL, DSC2, SPTN1, FLNA, COR1C, CADH1, DSG3) are involved in the structural cohesion of tissues as a part of anchoring junctions components or play roles in structural integrity of a cell (K2C8, K1C25, TBA1B). The most differently expressed female protein was ELMO1 that is involved in cytoskeletal rearrangements during phagocytosis. Second protein

accounting for female specific expression profile was SMGC (*Muc19*), which is important in the homeostasis of ocular mucus. MUC2 was also significantly female biased and is known from various mucus membrane-containing organs where it forms a protective barrier against particles or excludes bacteria from the inner mucus layer. CHIL3 is a glycoprotein that plays a role in inflammation and allergy. Female specific proteins also include e.g. enzymes such as CBR2, which is involved in xenobiotic metabolism, or AKC1H converting progesterone to 20-alpha-dihydroprogesteron.

The most abundant as well as the most male biased proteins in nasal mucosa were SCGB2B20 and ESP1. Expression of SCGB2B20 corroborates our previous results on the tear and saliva proteomes where their expression was abundant and male biased (Stopka et al., 2016;Stopkova et al., 2017). However, ESP1 (and also ESP22) transcripts were not detected in VNO or MOE. Interestingly, the nasal secretions also contained the male-biased group-B/central MUPs – MUP9 (FC=4.34, P=0.01), sMUP17 (FC=2.2, P=0.025) and other un-biased MUPs (e.g. MUP10) and OBPs depicted in Figure 3A. Analysis of male biased genes in String databases revealed significant interactions and participations in several processes e.g. hormone responses, responses to organonitrogen compound or complement and coagulation cascades. Some proteins are involved in detoxification or in antimicrobial defence, e.g. EST1C is involved in detoxification of xenobiotics, CATA protects cells from the toxic effects of hydrogenperoxide and PERM – myeloperoxidase with microbicidal activity.

To conclude, many of these sex-dimorphic proteins are involved in the preventive protection from bacteria, bactericidal activity and detoxification. Thus we further concentrated on the role of antimicrobial proteins in the regulation of lipocalins, which accounted for the majority of proteins and transcripts in our datasets. Furthermore, inter-individual variability in the abundance of lipocalins was high, thus, we tried to explain whether the level of variation correlates with other proteins.

Correlation with antimicrobial proteins (AMP)

Most mucosal tissues of the mouse produce peptides and proteins that physically break the membranes of bacteria. They are among the most expressed proteins in this study (e.g. BPIFB9B and BPIFB7 in Figure 3B) and thus we identified other AMPs with ontology searches in our data and performed multiple correlations to detect potential functional associations with lipocalins. These AMPs, for example include a natural antibiotics CAMP, that forms an amphipathic alpha-helix similar to other antimicrobial peptides, and functional studies demonstrated that CAMP is a potent antibiotics against gram-negative bacteria by inhibiting the growth of a variety of bacterial strains and is expressed by neutrophils and macrophages (Gallo et al., 1997). NGP (Neutrophilic granule protein) or ‘bectenecin’ – also belongs to cathelicidins, has a cathelicidin protein domain, and in our data, CAMP is highly correlated with NGP (r=0.95, p=0.027) and with LCN2 (r=0.95, p=0.04) and marginally with LYZ2 (r=0.92, p=0.07) on the level of individuals. LYZ2 is also active against a range of Gram-positive and Gram-negative bacteria. Specific antimicrobial activity has been demonstrated for the mouse lipocalin LCN2, which is up-regulated as a response to inflammation in mucosal tissues (Goetz et al., 2002;Flo et al., 2004), and which scavenges for catecholate-type siderophores that bacteria use to sequester free iron (Flo et al., 2004). LCN2 is equally present in male and female saliva (Stopka et al., 2016), tears (Stopkova et al., 2017) and nose in this study. The individual levels of

gene expression of *Lcn2, Ngp, Camp, Lyz2* are correlated in MOE (*Camp* vs. *Ngp*: r=0.98, p= 0.00005; *Camp* vs. *Lcn2*: r=0.78, p=0.004, *Camp* vs. *Lyz2*: r=0.95, p=0.007) and the same pattern is corroborated on the level of nasal proteome (Figure 4B). Thus, our hierarchical clustering based on the correlations between AMPs and lipocalins in Figure 4 revealed this particular functional group of proteins that are already known for their capacity to kill bacteria in order to regulate microbiota or to prevent pathogens entering the body. This is why we suggest that other identified sub-clusters may also be considered as functional units within a network of antimicrobial defense.

The most interesting result of our study was the finding that MUPs and OBPs cluster with several antimicrobial proteins. These include the bactericidal/permeability-increasing proteins - BPI (LeClair, 2003b;a) which are male-biased in the mouse saliva (Stopka et al., 2016), and it has been suggested that the products from defeated bacteria and from symbiotic microbiomes may be sexually dimorphic due to the sexually dimorphic expression of anti-microbial proteins. They may contribute to an existing pool of compounds that may be recognized as individual signals by which the mice recognize an individual's health (Zala et al., 2004;Zala et al., 2015). Here we tried to broaden this framework with our results on correlations with an idea that the upregulated lipocalins function as essential transporting devices that are necessary for the removal of the products of defeated bacteria and thus we expected positive correlations between lipocalins and AMPs. Each particular group of lipocalin transcripts (e.g. encoding OBPs, MUPs, LCN3 and LCN4, LCN13 and LCN14) seem to have few particular partners with which they are similarly regulated in VNO and MOE (Figure 4A, B) and correlated on the level of proteins (Figure 4C). For example, LCN3, LCN4, LCN16 correlate (on the level of p-adjusted<0.05) with BPIA1, BPIB6, and SOD3 (superoxide dismutase 3 is involved in the degradation of reactive oxygen species). WFDC proteins (i.e. 'Whey acidic proteins four disulphide core') were also shown to have anti-microbial properties (Scott et al., 2011) and the two members WFDC12 and WFDC18 are present in mouse saliva as proteins encoded by submandibular gland transcripts, i.e. *Wfdc12*, *Wfdc18* (Stopka et al., 2016). In exorbital lacrimal glands, we have detected *Wfdc12* and *Wfdc18* transcripts, but only WFDC18 was detected in tears. In MOE and VNO, we have detected *Wfdc1, Wfdc2, Wfdc3* and *Wfdc18* transcripts, whilst on the level of proteome we have detected only WFDC2 highly correlated with BPIB4 (r=0.88, p=0.0008), with the two cystatins CYTA (r=0.86, p=0.001), CYTB (r=0.65, p=0.04), and with OBP6 (r=0.88, p=0.0007). OBP6 as well as ESP1, however, were not detected on the level of VNO transcripts and only few *Obp6* transcripts were detected in MOE. They are most likely the products of other nasal glands or for example of those that are producing the tear proteome. They may be transported with mucosa to nasal cavity via naso-lacrimal ducts. OBP6 transcripts (*Obp6*) were not detected in nasal tissues (i.e. including the nasal-associated lymphoid tissue, MOE, and VNO) even in our previous qPCR study (Stopkova et al., 2016), thus it is likely an exogeneous protein in the nasal cavity proteome of the mouse.

AMPs and the variation in major urinary and odorant binding proteins

To our knowledge, urinary MUP variation is best explained by age and by various social factors (Thoß et al., 2015;Enk et al., 2016;Thoss et al., 2016). Here we show, that nasal MUPs correlate with AMPs and with other MUPs (sMUP9 vs. BPIFB5: r=0.88, p=0.0008, sMUP9 vs. MUP5: r=0.77, p=0.009; sMUP17 vs. BPIB1: r=0.83,

p=0.003; MUP10 vs. BPIFB7: r=0.66, p=0.037) on the level of proteome. On the level of transcripts, we have only detected group-A *Mup*s in MOE and VNO. They were also correlated with genes for AMPs. For example in MOE, *Mup4* was correlated with *Bifb9b* (r=0.78, p=0.004) and with *Obp*s (*Mup4* vs. any *Obp*: r>0.6, p=0.02 to 0.005). Similarly *Mup5* was significantly correlated with all *Obp*s (r>0.85, p<0.001). *Obp*s in MOE were highly correlated with the bacterial receptor *Pglyrp1* (r~0.9, p<0.0001). In VNO, the trend in correlations was slightly less obvious but, for example, all *Obp*s correlated with *Mup4* (r~0.9, p<0.0002) and with *Bpia1* (r>0.66, p<0.02) and with *Bpifb3* (r>0.6, p<0.05). All combinations of correlation coefficients and p-adjusted values are provided in the Supplementary dataset and visualised in Figure 4.

Discussion

In this study, we have provided the main olfactory (MOE) and vomeronasal (VNO) transcriptomes of the wild-derived house mice, *M. m. musculus*. At the same time we have generated the proteome of the nasal cavity with the state-of-the-art label-free LC-MS/MS techniques to provide a further support on the expression of several novel genes on the level of protein and to obtain evidence on sexual dimorphisms that remained undetected with previous – mainly RNA/RNAseq-based – techniques.

Sexual dimorphism and highly expressed transcripts

We compared the transcriptomes from VNO and MOE to search for differences between males and females that may yield potential differences in the detection of sex specific signals and odour-based behavioural patterns. However, the level of the detected sex dimorphisms was extremely low on the level of transcripts and the X or Y-chromosome linked transcripts were mostly responsible for this variation. At the same time, we provide evidence that several lipocalin coding transcripts (e.g. *Lcn13, Lcn14, Obp*s) belong to the most expressed genes in both tissues. We have detected a total of 19 lipocalin transcripts in VNO and 20 lipocalin transcripts in MOE. OBP coding transcripts (i.e. sensu (Stopková et al., 2009;Stopkova et al., 2014;Stopkova et al., 2016)) were present in both tissues (*Obp1, Obp2, Obp5, Obp7, Obp8*) as well as *Obp3-ps* pseudogene, whilst *Obp6* was absent in VNO and only small numbers of reads were detected in MOE. We have also detected *Mup4, Mup5* in VNO and MOE and on top, MOE also expressed *Mup-ps22* and *Mup6*. MOE and VNO equally expressed *Lcn2, Lcn3, Lcn4, Lcn11, Lcn13,* and *Lcn14*. Thus, given lipocalins seem to be equally important for individuals of both sex. As proteins, they may be essential either as the transporting devices in MOE and VNO that scavenge for potentially harmful ligands (i.e. evidence provided for OBPs (Grolli et al., 2006)), transport pheromones to the vicinity of neurons, and that drive the ligands for internalization in lysozomes (Strotmann and Breer, 2011). They may also function as the cleaning devices that remove superfluous background odorants to make the olfactory tissues continuously functional. It makes perfectly sense that it is a mixture of different lipocalins, because they have different biochemical properties such that OBPs are less hydrophobic than MUPs or LCNs (Stopkova et al., 2014;Stopkova et al., 2016). Different transporters may scavenge for a wider spectrum of ligands and of different types including hydrophobic pheromones as well as the harmful organic compounds such as 4-Hydroxynon-2-enal (HNE), which is a product of lipid peroxidation and causes chronic inflammation in mucosal tissues (Grolli et al., 2006).

The nasal cavity proteome

The level of sexual dimorphism in nasal secretions was surprisingly high with 8.7% of proteins being male biased and 8.1% proteins being female biased. This is similar to the level of sexual dimorphism that we recently determined in tears with 7% of proteins being male biased and 7% proteins being female biased (Stopkova et al., 2017). Some proteins were present in nasal secretions but not coded by genes in MOE and VNO. These may include for example OBP6, and the significantly male-biased (i.e. not male-unique) exocrine gland-secreted peptides ESP1 and ESP22. ESP1 is produced by the mouse lacrimal glands, secreted with tears and when experimentally transferred to the female vomeronasal organ, it stimulates V2R-expressing vomeronasal chemosensory neurons, and thus elicits an electrical response (Kimoto et al., 2005;Kimoto et al., 2007). In wild house mice they are male unique in tears but male-biased on the level of the lacrimal gland transcriptome (Stopkova et al., 2017). This study shows that ESP1 along with ESP22 are also present in females, though in lower quantities, and they may be involved in other as yet unknown functions. ESP1 has three α-helices with two helices being negatively charged and one being positively charged. This structural amphypathy fits the description of antimicrobial peptides which is similar to CAMP/CRAMP (Stopka et al., 2016). Thus it is possible that nasal ESPs (i.e. including ESP1 and ESP22) are involved in the host-defense against bacteria. Similarly, OBP6 was also detected as abundant in tears and as *Obp6* in the lacrimal gland transcriptome (Stopkova et al., 2017) as well as in the nasal secretions on the level of proteins but not on the level of VNO and MOE transcripts in this study. Interestingly, the nasal secretions also contained male-biased group-B/central MUPs depicted in Figure 3D.

The most interesting result of this study is evidence that among the most expressed proteins of the nasal secretions are OBPs, MUPs, LCNs and antimicrobial proteins. When the pattern of lipocalin expression is hierarchically clustered, their levels are correlated with AMPs, Figure 4. It seems likely that this clustered organization underpins their roles in removing organic products of bacterial degradation and of those of the host. Previously we have presented the 'Toxic waste hypothesis' which states that MUPs, OBPs, and some other lipocalins are involved in the process of detoxification as the transporting devices that remove potentially harmful molecules from mucosal tissues, and that these ligands may become signals if their level correlates with individual quality (Stopková et al., 2009;Stopkova et al., 2014). This paper builds upon our previous studies which provided evidence that MUPs and OBPs may occur in the two different levels of expression as highly expressed in some tissues (i.e. central MUPs in the liver) and with lower levels of expression in many other – e.g. (Stopka et al., 2016;Stopkova et al., 2016;Stopkova et al., 2017), thus being in the proteome darkspace, where they may function within their ancestral roles as transporting devices of potentially harmful substances. Their emergence from the proteome darkspace may be facilitated by means of evolution of tissue-specific regulation if a candidate lipocalin is selected for a new function when a new binding capacity is acquired for a ligand that, though harmful, my signal individual quality and correlates with individual status. This study also builds upon several previous studies which provided evidence that MUPs (Kwak et al., 2016) and OBPs (Grolli et al., 2006) bind toxic waste, and that mice recognize infected males on the basis of their odors (Zala et al., 2004;Zala et al., 2015). Chemodetection of microorganism-associated molecular patterns (MAMPs) occurs at many places in the body including specific sets of chemosensory neurons in the mammalian nose (Bufe and Zufall, 2016) and formyl peptide receptor-like proteins in VNO, which provide sensitivity to

disease/inflammation-related ligands (Riviere et al., 2009). In our data, we have detected the five formyl-peptide receptors – *Fpr-rs3*, *Fpr-rs6, Fpr-rs7, Fpr2,* and *Fpr1* in VNO whilst in MOE we have detected just *Fpr1* and *Fpr2* (Supplementary dataset – raw data).

To add, nasal lipocalins may contribute to removing various ligands after sensing which is supported by high correlation levels between lipocalin expression (MUPs, OBPs) in MOE and Peptidoglycan recognition protein 1 (PGRP1/*Pglyrp1*, Figure 4B) which activates bacterial tool-component systems (Royet et al., 2011). In nasal secretions, most AMPs and lipocalins were non-dimorphic. Here we conclude for the first time that individual variation in the abundance of lipocalins (e.g. MUPs, OBPs) may be caused by their dependence on the expression of a clustered network of antimicrobial peptides that regulate microbiota and pathogens.

Competing interests
The authors declare that they have no competing interests.

Author contributions statements
PS, RS and BK wrote the main manuscript text, BK helped to annotate proteomic datasets and collected nasal samples. All authors have reviewed the manuscript.

Acknowledgement
This research was supported by the project BIOCEV (CZ.1.05/1.1.00/02.0109) and National Program for Sustainability II (LQ1604) from the Ministry of Education, Youth and Sports. The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. We are also grateful to Helena Uhlířová for her careful and patient lab assistance and to Karel Harant and Pavel Talacko from the Mass Spectrometry and Proteomics Service Laboratory, Faculty of Science, Charles University for performing the LC-MS/MS run.

References

Ben-Shaul, Y., Katz, L.C., Mooney, R., and Dulac, C. (2010). In vivo vomeronasal stimulation reveals sensory encoding of conspecific and allospecific cues by the mouse accessory olfactory bulb. *Proc Natl Acad Sci U S A* 107**,** 5172-5177.

Bergan, J.F., Ben-Shaul, Y., and Dulac, C. (2014). Sex-specific processing of social cues in the medial amygdala. *Elife* 3**,** e02743.

Bolstad, B.M., Irizarry, R.A., Astrand, M., and Speed, T.P. (2003). A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. *Bioinformatics* 19**,** 185-193.

Buck, L., and Axel, R. (1991). A novell multigene family may encode odorant receptors: A molecular basis for odor recognition. *Cell* 65**,** 175-187.

Buck, L.B. (2000). The molecular architecture of odor and pheromone sensing in mammals. *Cell* 100**,** 611-618.

Bufe, B., and Zufall, F. (2016). The sensing of bacteria: emerging principles for the detection of signal sequences by formyl peptide receptors. *Biomol Concepts* 7**,** 205-214.

Cichy, A., Ackels, T., Tsitoura, C., Kahan, A., Gronloh, N., Sochtig, M., Engelhardt, C.H., Ben-Shaul, Y., Muller, F., Spehr, J., and Spehr, M. (2015). Extracellular

pH Regulates Excitability of Vomeronasal Sensory Neurons. *J Neurosci* 35**,** 4025-4039.

Cox, J., Hein, M.Y., Luber, C.A., Paron, I., Nagaraj, N., and Mann, M. (2014). Accurate proteome-wide label-free quantification by delayed normalization and maximal peptide ratio extraction, termed MaxLFQ. *Mol Cell Proteomics* 13**,** 2513-2526.

Crawley, M.J. (2007). *The R Book.* Wiley Publishing.

Enk, V.M., Baumann, C., Thoss, M., Luzynski, K.C., Razzazi-Fazeli, E., and Penn, D.J. (2016). Regulation of highly homologous major urinary proteins in house mice quantified with label-free proteomic methods. *Mol Biosyst* 12**,** 3005-3016.

Flo, T.H., Smith, K.D., Sato, S., Rodriguez, D.J., Holmes, M.A., Strong, R.K., Akira, S., and Aderem, A. (2004). Lipocalin 2 mediates an innate immune response to bacterial infection by sequestrating iron. *Nature* 432**,** 917-921.

Gallo, R.L., Kim, K.J., Bernfield, M., Kozak, C.A., Zanetti, M., Merluzzi, L., and Gennaro, R. (1997). Identification of CRAMP, a cathelin-related antimicrobial peptide expressed in the embryonic and adult mouse. *J Biol Chem* 272**,** 13088-13093.

Gentleman, R.C., Carey, V.J., Bates, D.M., Bolstad, B., Dettling, M., Dudoit, S., Ellis, B., Gautier, L., Ge, Y., Gentry, J., Hornik, K., Hothorn, T., Huber, W., Iacus, S., Irizarry, R., Leisch, F., Li, C., Maechler, M., Rossini, A.J., Sawitzki, G., Smith, C., Smyth, G., Tierney, L., Yang, J.Y., and Zhang, J. (2004). Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol* 5**,** R80.

Goetz, D.H., Holmes, M.A., Borregaard, N., Bluhm, M.E., Raymond, K.N., and Strong, R.K. (2002). The Neutrophil Lipocalin NGAL Is a Bacteriostatic Agent that Interferes with Siderophore-Mediated Iron Acquisition. *Molecular Cell* 10**,** 1033-1043.

Grolli, S., Merli, E., Conti, V., Scaltriti, E., and Ramoni, R. (2006). Odorant binding protein has the biochemical properties of a scavenger for 4-hydroxy-2-nonenal in mammalian nasal mucosa. *FEBS Journal* 273**,** 5131-5514.

Hurst, J.L., Beynon, R.J., Armstrong, S.D., Davidson, A.J., Roberts, S.A., Gomez-Baena, G., Smadja, C.M., and Ganem, G. (2017). Molecular heterogeneity in major urinary proteins of Mus musculus subspecies: potential candidates involved in speciation. *Sci Rep* 7**,** 44992.

Ibarra-Soria, X., Levitin, M.O., Saraiva, L.R., and Logan, D.W. (2014). The Olfactory Transcriptomes of Mice. *Plos Genetics* 10.

Janotova, K., and Stopka, P. (2011). The level of major urinary proteins is socially regulated in wild Mus musculus musculus. *J Chem Ecol* 37**,** 647-656.

Jemiolo, B., Andreolini, F., Xie, T.-M., Wiesler, D., and Novotny, M. (1989). Puberty-affecting synthetic analogs of urinary chemosignals in the house mouse, Mus domesticus. *Physiology & Behavior* 46**,** 293-298.

Jemiolo, B., Harvey, S., and Novotny, M. (1986). Promotion of the Whitten effect in female mice by synthetic analogs of male urinary constituents. *PNAS* 83**,** 4576-4579.

Jemiolo, B., and Novotny, M.V. (1994). Inhibition of sexual maturation in juvenile female and male mice by a chemosignal of female origin. *Physiology and Behavior* 55**,** 519-522.

Jemiolo, B., Xie, T.-M., and Novotny, M. (1992). Urine marking in male mice: Responses to natural and synthetic chemosignals. *Physiology and Behavior* 52**,** 521-526.

Kahan, A., and Ben-Shaul, Y. (2016). Extracting Behaviorally Relevant Traits from Natural Stimuli: Benefits of Combinatorial Representations at the Accessory Olfactory Bulb. *PLoS Comput Biol* 12**,** e1004798.

Kimoto, H., Haga, S., Sato, K., and Touhara, K. (2005). Sex-specific peptides from exocrine glands stimulate mouse vomeronasal sensory neurons. *Nature* 437**,** 898-901.

Kimoto, H., Sato, K., Nodari, F., Haga, S., Holy, T.E., and Touhara, K. (2007). Sex- and strain-specific expression and vomeronasal activity of mouse ESP family peptides. *Curr Biol* 17**,** 1879-1884.

Knopf, J.L., Gallagher, J.F., and Held, W.A. (1983). Differential, multihormonal regulation of the mouse major urinary protein gene family in the liver. *Mol. Cell. Biol.* 3**,** 2232-2240.

Kwak, J., Strasser, E., Luzynski, K., Thoss, M., and Penn, D.J. (2016). Are MUPs a Toxic Waste Disposal System? *PLoS One* 11**,** e0151474.

Leclair, E.E. (2003a). Four BPI (bactericidal/permeability-increasing protein)-like genes expressed in the mouse nasal, oral, airway and digestive epithelia. *Biochem Soc Trans* 31**,** 801-805.

Leclair, E.E. (2003b). Four reasons to consider a novel class of innate immune molecules in the oral epithelium. *J Dent Res* 82**,** 944-950.

Leinders-Zufall, T., Lane, A.P., Puche, A.C., Ma, W., Novotny, M.V., Shipley, M.T., and Zufall, F. (2000). Ultrasensitive pheromone detection by mammalian vomeronasal neurons. *Nature* 405**,** 792-796.

Logan, D.W., Marton, T.F., and Stowers, L. (2008). Species Specificity in Major Urinary Proteins by Parallel Evolution. *PLoS ONE* 3**,** doi: 10.1371/journal.pone.0003280.

Ma, W., Miao, Z., and Novotny, M.V. (1999). Induction of Estrus in Grouped Female Mice (Mus domesticus) by Synthetic Analogues of Preputial Gland Constituents

10.1093/chemse/24.3.289. *Chem. Senses* 24**,** 289-293.

Moss, R.L., Flynn, R.E., Shen, X., Dudley, C., Shi, J., and Novotny, M. (1997). Urine-Derived Compound Evokes Membrane Responses in Mouse Vomeronasal Receptor Neurons. *Journal of Neurophysiology* 77**,** 2856-2862.

Mudge, J.M., Armstrong, S.D., Mclaren, K., Beynon, R.J., Hurst, J.L., Nicholson, C., Robertson, D.H., Wilming, L.G., and Harrow, J.L. (2008). Dynamic instability of the major urinary protein gene family revealed by genomic and phenotypic comparisons between C57 and 129 strain mice. *Genome Biol* 9**,** R91.

Novotny, M.V., Jemiolo, B., Wiesler, D., Ma, W., Harvey, S., Xu, F., Xie, T., and Carmack, M. (1999a). A unique urinary constituent, 6-hydroxy-6-methyl-3-heptanone, is a pheromone that accelerates puberty in female mice. *Chem. Biol.* 6**,** 377-383.

Novotny, M.V., Ma, W., Zidek, L., and Daev, E. (1999b). "Recent biochemical insights into puberty acceleration, estrus induction, and puberty delay in the house mouse," in *Advances in Chemical Communication in Vertebrates,* eds. R.E. Johnston, D. Muller-Schwarze & P. Sorensen. (New York: Plenum Press), 99-116.

Pavelka, N., Fournier, M.L., Swanson, S.K., Pelizzola, M., Ricciardi-Castagnoli, P., Florens, L., and Washburn, M.P. (2008). Statistical similarities between

transcriptomics and quantitative shotgun proteomics data. *Mol Cell Proteomics* 7**,** 631-644.

Pavelka, N., Pelizzola, M., Vizzardelli, C., Capozzoli, M., Splendiani, A., Granucci, F., and Ricciardi-Castagnoli, P. (2004). A power law global error model for the identification of differentially expressed genes in microarray data. *BMC Bioinformatics* 5**,** 203.

Riviere, S., Challet, L., Fluegge, D., Spehr, M., and Rodriguez, I. (2009). Formyl peptide receptor-like proteins are a novel family of vomeronasal chemosensors. *Nature* 459**,** 574-577.

Rodriguez, J., Gupta, N., Smith, R.D., and Pevzner, P.A. (2008). Does trypsin cut before proline? *J Proteome Res* 7**,** 300-305.

Royet, J., Gupta, D., and Dziarski, R. (2011). Peptidoglycan recognition proteins: modulators of the microbiome and inflammation. *Nat Rev Immunol* 11**,** 837-851.

Sam, M., Vora, S., Malic, B., Ma, W., Novotny, M.V., and Buck, L.B. (2001). Odorants may arouse instinctive behaviours. *Nature* 412**,** 142.

Sampsell, B., and Held, W. (1985). Variation in the major urinary protein multigene family in wild-derived mice. *Genetics* 109**,** 549-568.

Scott, A., Weldon, S., and Taggart, C.C. (2011). SLPI and elafin: multifunctional antiproteases of the WFDC family. *Biochem Soc Trans* 39**,** 1437-1440.

Sharrow, S.D., Novotny, M.V., and Stone, M.J. (2003). Thermodynamic analysis of binding between mouse major urinary protein-I and the pheromone 2-sec-butyl-4,5-dihydrothiazole. *Biochemistry* 42**,** 6302-6309.

Sharrow, S.D., Vaughn, J.L., Žídek, L., Novotny, M.V., and Stone, M.J. (2002). Pheromone binding by polymorphic mouse major urinary proteins. *Protein Science* 11**,** 2247-2256.

Shiao, M.S., Chang, A.Y., Liao, B.Y., Ching, Y.H., Lu, M.Y., Chen, S.M., and Li, W.H. (2012). Transcriptomes of mouse olfactory epithelium reveal sexual differences in odorant detection. *Genome Biol Evol* 4**,** 703-712.

Stopka, P., Janotova, K., and Heyrovsky, D. (2007). The advertisement role of major urinary proteins in mice. *Physiology & Behavior* 91**,** 667-670.

Stopka, P., Kuntova, B., Klempt, P., Havrdova, L., Cerna, M., and Stopkova, R. (2016). On the saliva proteome of the Eastern European house mouse (Mus musculus musculus) focusing on sexual signalling and immunity. *Sci Rep* 6**,** 32481.

Stopkova, R., Dudkova, B., Hajkova, P., and Stopka, P. (2014). Complementary roles of mouse lipocalins in chemical communication and immunity. *Biochemical Society Transactions* 42**,** 893-898.

Stopková, R., Hladovcová, D., J., K., Vyoral, D., and Stopka, P. (2009). Multiple roles of secretory lipocalins (MUP, OBP) in mice. *Folia Zool.* 58**,** 29-40.

Stopkova, R., Klempt, P., Kuntova, B., and Stopka, P. (2017). On the tear proteome of the house mouse (Mus musculus musculus) in relation to chemical signalling. *PeerJ* 6:e3541.

Stopková, R., Stopka, P., Janotová, K., and Jedelsky, P.L. (2007). Species-specific expression of major urinary proteins in the house mice (Mus musculus musculus and Mus musculus domesticus). *J Chem Ecol* 33**,** 861-869.

Stopkova, R., Vinkler, D., Kuntová, B., Sedo, O., Albrecht, T., Suchan, J., Dvorakova-Hortova, K., Zdrahal, Z., and Stopka, P. (2016). Mouse lipocalins (MUP, OBP, LCN) are co-expressed in tissues involved in chemical communication. *Frontiers in Ecology and Evolution* 4.

Strotmann, J., and Breer, H. (2011). Internalization of odorant-binding proteins into the mouse olfactory epithelium. *Histochem Cell Biol* 136**,** 357-369.

Thoss, M., Enk, V., Yu, H., Miller, I., Luzynski, K.C., Balint, B., Smith, S., Razzazi-Fazeli, E., and Penn, D.J. (2016). Diversity of major urinary proteins (MUPs) in wild house mice. *Sci Rep* 6**,** 38378.

Thoß, M., Luzynski, K., Ante, M., Miller, I., and Penn, D.J. (2015). Major urinary protein (MUP) profiles show dynamic changes rather than individual 'barcode' signatures. *Frontiers in Ecology and Evolution* 3.

Timm, D.E., Baker, L.J., Mueller, H., Zidek, L., and Novotny, M.V. (2001). Structural basis of pheromone binding to mouse major urinary protein (MUP-I). *Protein Science* 10**,** 997-1004.

Zala, S.M., Bilak, A., Perkins, M., Potts, W.K., and Penn, D.J. (2015). Female house mice initially shun infected males, but do not avoid mating with them. *Behavioral Ecology and Sociobiology* 69**,** 715-722.

Zala, S.M., Potts, W.K., and Penn, D.J. (2004). Scent-marking displays provide honest signals of health and infection. *Behavioral Ecology* 15**,** 338-344.

Zidek, L., Stone, M.J., Lato, S.M., Pagel, M.D., Miao, Z., Ellington, A.D., and Novotny, M.V. (1999). NMR Mapping of the Recombinant Mouse Major Urinary Protein I Binding site Occupied by the Pheromone 2-*sec*-Butyl-4,5-dihydrothiazole. *Biochemistry* 38**,** 9850-9861.

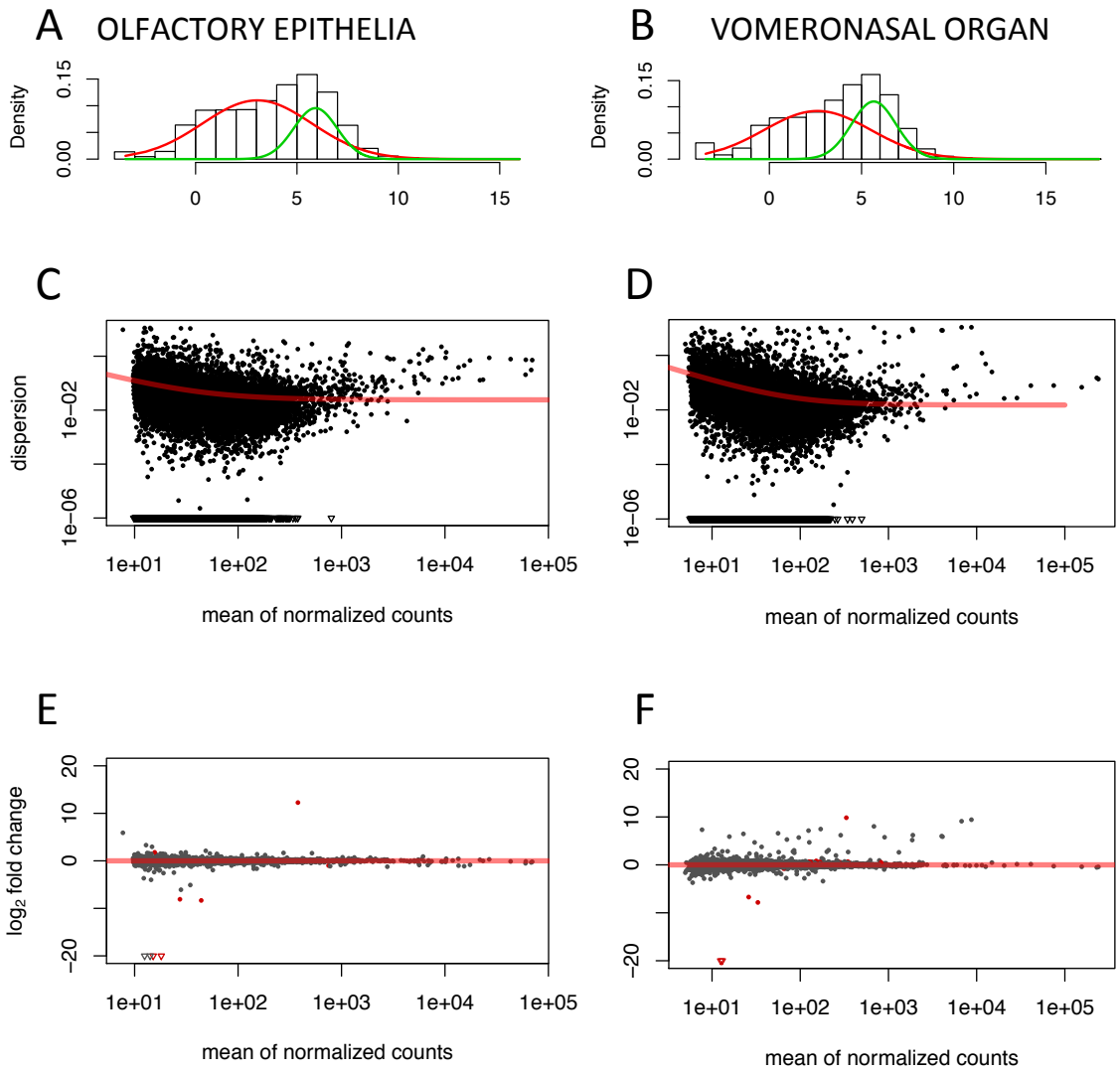


Figure 1: **Result outputs of RNA-seq analysis.** Fitting mixture distributions reveals the two groups of low and high expression data from MOE (A) and VNO (B). The two solid curves shown in the plots (red, green) correspond to the individual Gaussian density components in the mixture distribution, each scaled by the estimated probability of an observation being drawn from that component distribution. Dispersion plots (C, D) show the decreasing variation in signal intensities, and demonstrate that the MOE and VNO transcripts with lower number of reads have a higher dispersion. The MA plots in C demonstrate that the level of sexual dimorphism is extremely low in MOE and VNO with only few data points (red) being sexually dimorphic. X-axis represent the basal mean of number of reads whilst the Y-axis represent particular fold changes.

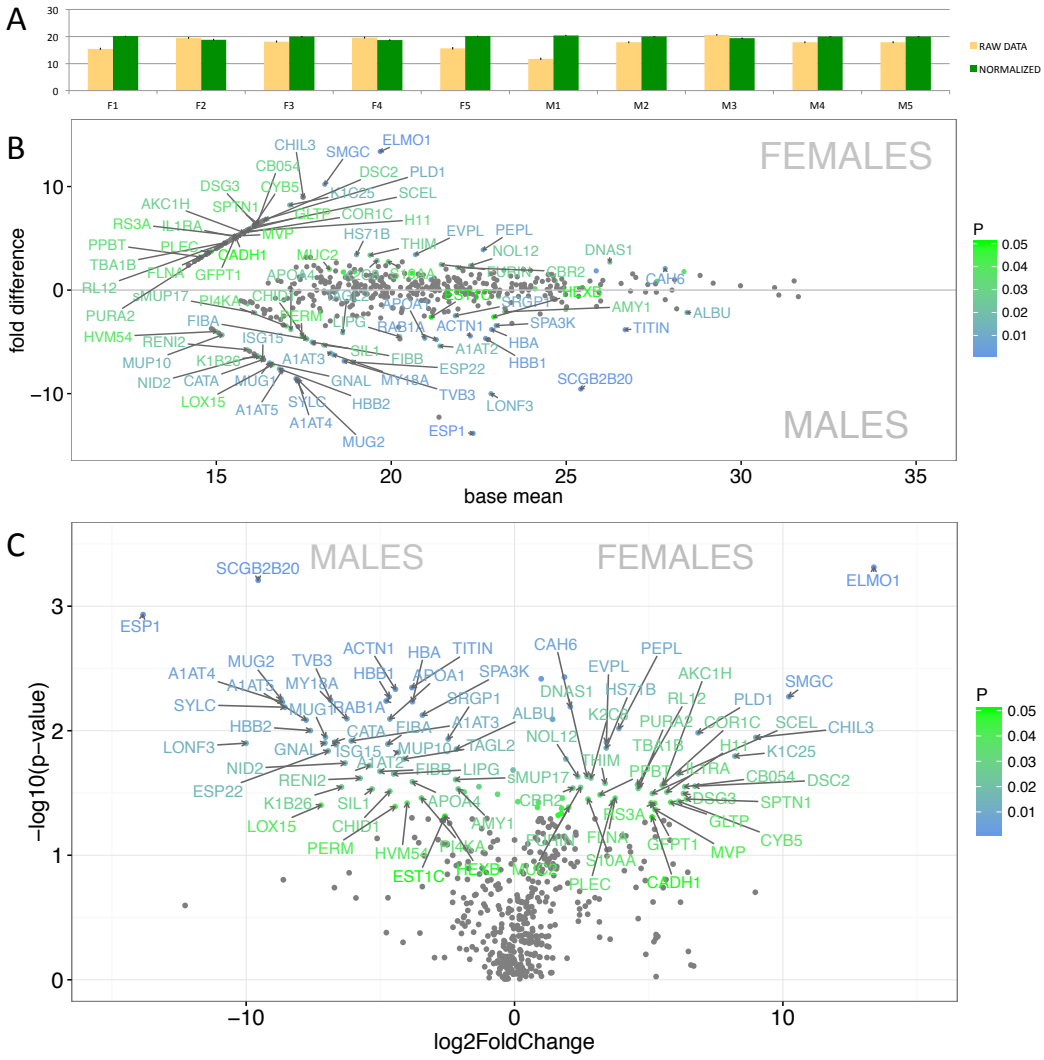


Figure 2: **Graphical representation of the mean protein signal intensities from LC-MS/MS (X axis) and of particular fold differences between males and females.** Before normalization, the data revealed some variation between individuals (A – yellow bars). However, after the quantile-normalization procedure (A – green bars), the mean value and standard error bars show almost no variation between the samples. Significant differentially-expressed proteins are demonstrated with the MA plots (B). PLGEM model was involved in testing the differences in normalized signal values between males and females (B). The level of significance (males vs. females) is scaled from green (P<0.05) to blue (P<0.01) and only the data points with FC>2 are annotated with protein names. The x-axis represent the basal mean of signal intensities in B. The dependence of particular fold changes on p-values is provided using the volcano plots in C.

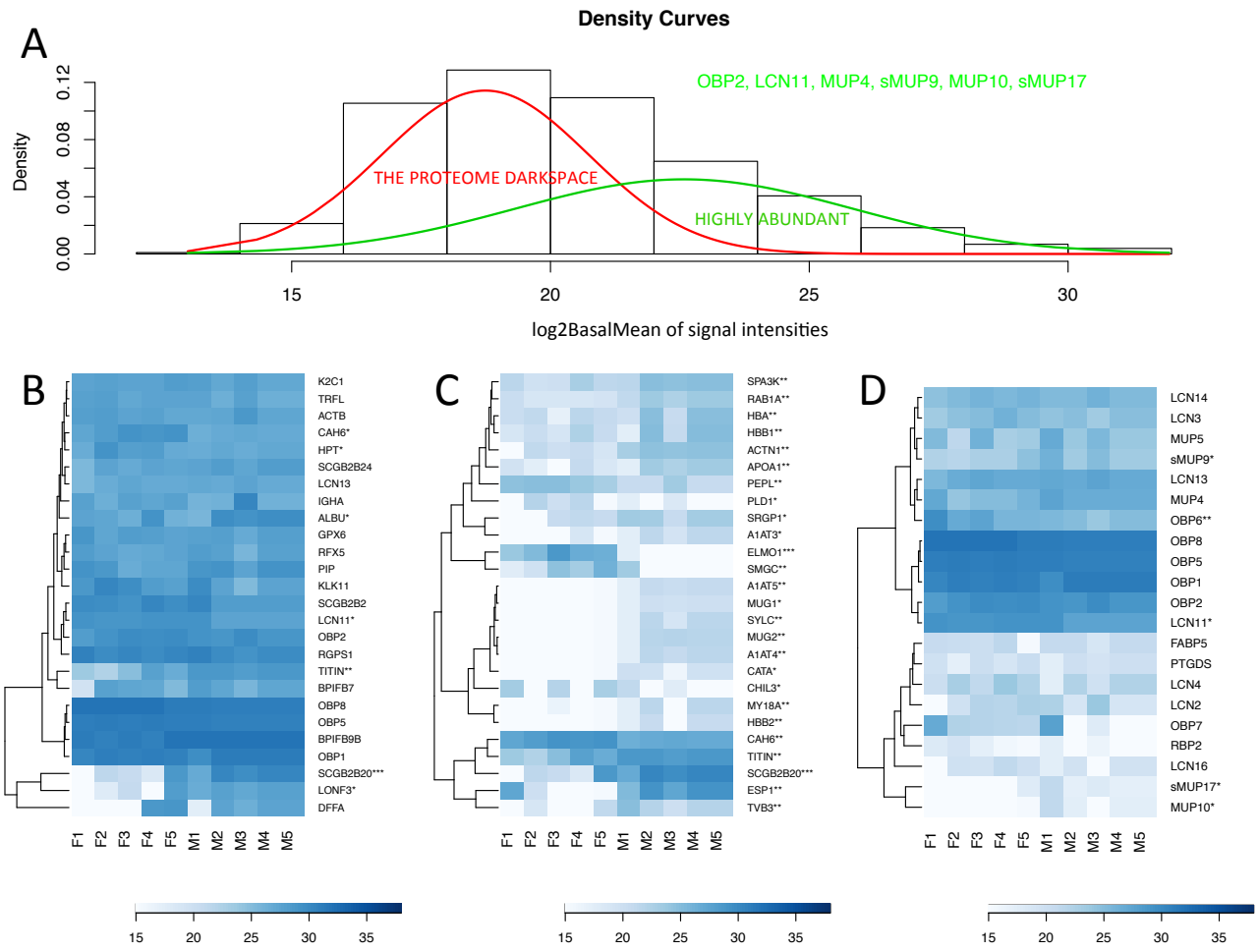


Figure 3: Analysis of the nasal cavity proteome. Graphical representations of protein signal distributions (A) reveal the two groups of high protein-abundance data (green curve) and low abundance data here coined as the 'proteome darkspace'. Similarities between proteins and individuals were detected with a hierarchical clustering method in heatmaps using complete linkage and Euclidean distance: (B) the top 5% of highly expressed proteins include e.g. LCN11, LCN13, OBP1, OBP2, OBP5, OBP8; (B) the top 5% of the most significant sexually dimorphic proteins ($p < 0.01$) include e.g. ESP1 and SCGB2B20. There is a notable variation between individuals in protein abundances (B, C, D). Note that the expression of most lipocalins is non-dimorphic with the exception of LCN11, and the group-B/central MUPs - sMUP9, MUP10 and sMUP17. Asterisks represent: * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$).

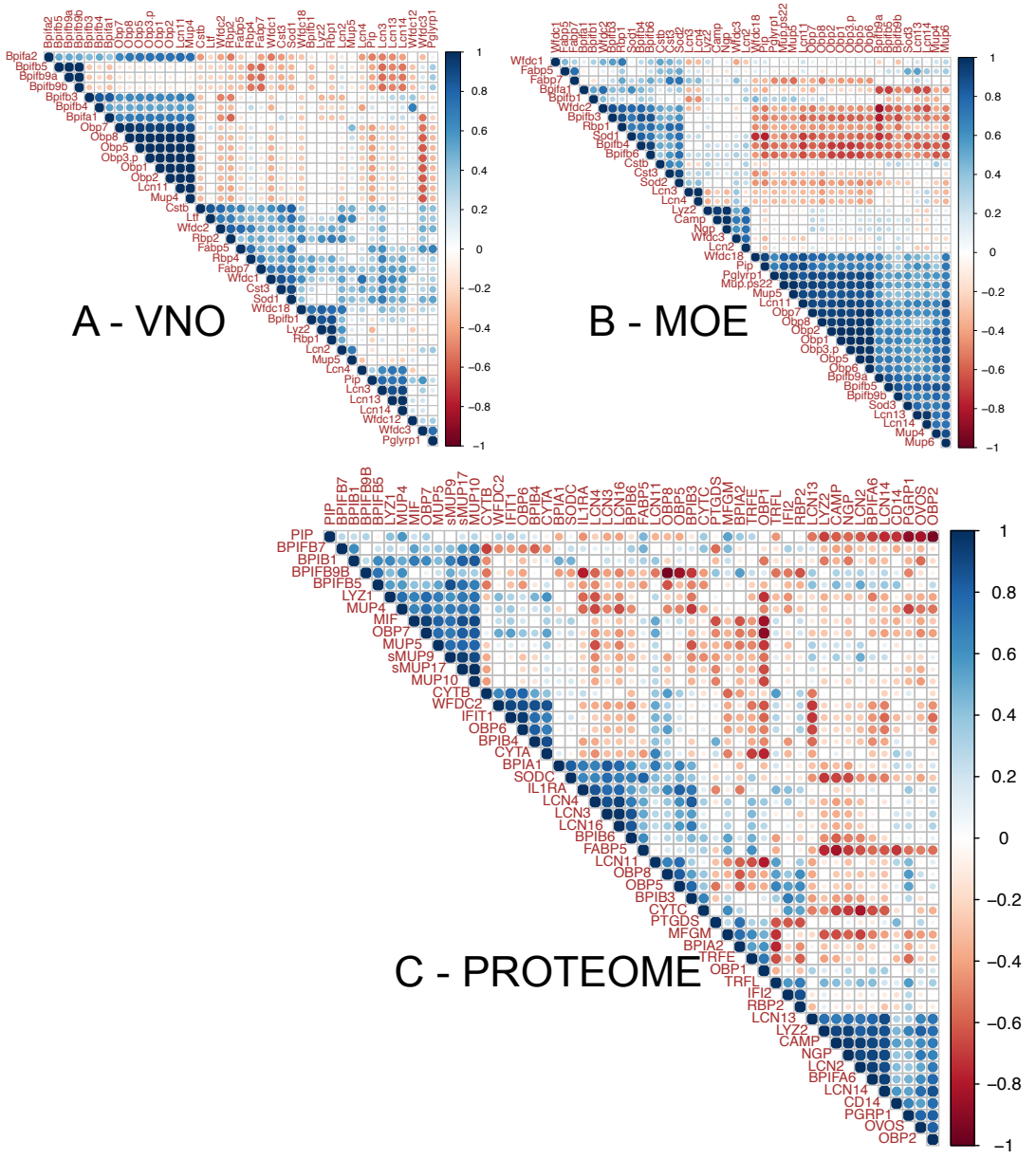


Figure 4: Correlation between lipocalins and antimicrobial proteins. We compared individual patterns of protein abundances. The three multiple correlation plots (corr. from -1 to 1 scaled from red to blue) – produced by hierarchical clustering with complete linkage method and Euclidean distance in A (VNO), B (MOE), and C (the nasal cavity proteome) – demonstrate that MUPs and OBPs reach the highest correlation with the levels of particular BPI proteins. Levels of *Bpifa1, Bpifa2, Bpifb3, Bpifb4* significantly correlate with *Obp1, Obp2, Obp7, Obp8, Lcn11,* and *Mup4* in VNO (A). Levels of *Bpifb9a, Bpifb9b,* and *Wfdc18* correlate with all *Obps, Mup4, Mup5,* and *Mup6* in MOE (B). On the level of proteome (C), MUPs (i.e. central sMUP9, MUP10, sMUP17, and outlier – MUP4 and MUP5) and OBP7 are correlated with antimicrobial LYZ1, BPIB1, BPIFB5, and to some extent with BPIFB7 and BPIFB9B. OBP2, LCN13, LCN14, and the bacterial-siderophore scavenging LCN2 are correlated with BPIFA6, LYZ2, and natural antibiotics CAMP (cathelicidin) and NGP (bectenecin). LCN3, LCN4, LCN16 are correlated with BPIA1 and BPIB6. The bigger the circle – the smaller is particular p-value ($p<0.05$).